NEUROPROTECTIVE EFFECT OF DIOSMIN ON ARSENIC TRIOXIDE INDUCED NEUROTOXICITY IN ALBINO WISTAR RATS

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1. INTRODUCTION

In neuroscience experimentation, neurotoxins were generally considered as exogenous substances, administered in order to lesion a particular neural phenotype (i.e., dopaminergic nerves) or subset of neurons with common features (i.e., those with kainate receptors). This narrow definition may have been valid when selective neurotoxins were first discovered approximately 50 years ago, but the concept of a neurotoxin is much more global today.[1]

Evolution of the science of neurotoxins has broadened the term to cover endogenous substances and metabolites, capable of initiating apoptotic, necrotic events leading to neuronal cell death. There are many pathways capable of provoking neuronal cell injury by disrupting normal cellular processes leading abnormal neural function that can impact neuronal interactions and incite events encompassed in psychiatric and substance use disorders.

Nosology of CNS disorders now considers the putative role of environmental factors and interplay with genes. Accordingly, in this context, the terms "neurotoxins and neurotoxicity" are more global and now encompass a broad spectrum of indigenous neuronal actions - all part of the evolution of this area of neuroscience. Substances that damage astrocytes - satellite supportive cells for neurons - also produce secondary neurotoxicity.

Loss of satellite cells leads to loss of neuronal cells. Therefore, astrocytic cytotoxins are deservedly included in the term 'neurotoxin'.

Another consideration that somewhat clouds the definition of a neurotoxin is the observed programmed cell death that occurs during neuronal ontogeny. If a substance is released by one nerve to promote apoptosis in extraneous neuronal progenitor cells, the outcome is "normalcy". One can only wonder if it is proper to regard the chemical which signals another nerve to die, to produce normalcy, as a neurotoxin.

A final consideration is the fact that a small number of repeated treatments with specific substances can produce long-term alterations in behavior, so that exaggerated behavioral effects are produced when the challenge substance is administered again, even a year or more after the initial short-term series of treatment. In this scenario there were seemingly no changes in morphology, changes in histochemical markers and numbers of receptors, nor neurotransmitter content or amounts of neurotransmitter released on stimulation.

Specifically, a short course of treatment with an exquisitely low dose of the DA D2 agonist quinpirole produced life-long enhancement of D2 agonist-mediated behaviors with no apparent change in any biochemical, histochemical or morphological index (Kostrzewa, 1995; Kostrzewa et al., 2003; 2004). This would be encompassed in the term 'neurotoxicity', because there is a change from normalcy.

Neurotoxicity occurs when the exposure to natural or artificial toxic substances, which are called neurotoxins, alters the normal activity of the nervous system in such a way as to cause damage to nervous tissue. This can eventually disrupt or even kill neurons, key cells that transmit and process signals in the brain and other parts of the nervous system.[2]

Neurotoxicity can result from exposure to substances used in chemotherapy, radiation treatment, drug therapies, certain drug abuse, and organ transplants, as well as exposure to heavy metals, certain foods and food additives, pesticides, industrial and/or cleaning solvents, cosmetics, and some naturally occurring substances.

Symptoms may appear immediately after exposure or be delayed. They may include limb weakness or numbness, loss of memory, vision, and/or intellect, uncontrollable obsessive and/or compulsive behaviors, delusions, headache, cognitive and behavioral problems and sexual dysfunction. Individuals with certain disorders may be especially vulnerable to neurotoxins.[3]

The name implies the role of a neurotoxin, although the term neurotoxic may be used more loosely to describe states that are known to cause physical brain damage but where no obvious neurotoxin has been identified.

The term neurotoxic is used to describe a substance, condition or state that damages the nervous system and/or brain, usually by killing neurons. The term is generally used to describe a condition or substance that has been shown to result in observable physical damage.

The presence of neurocognitive deficits alone are not usually considered sufficient evidence of neurotoxicity, as many substances exist which may impair neurocognitive performance without resulting in the death of neurons. This may be due to the direct action of the substance, with the impairment and neurocognitive deficits being temporary, and resolving when the substance is metabolised from the body.[4]

In some cases the level or exposure-time may be critical, with some substances only becoming neurotoxic in certain doses or time periods. Some of the most common naturally occurring brain toxins that lead to neurotoxicity as a result of excessive dosage are Beta amyloid (Aβ), Glutamate and Oxygen radicals.[5]

When present in high concentrations they can lead to neurotoxicity and death (apoptosis). Some of the symptoms that result from cell death include loss of motor control, cognitive deterioration and autonomic nervous system dysfunction. Additionally, neurotoxicity has been found to be a major cause of neurodegenerative diseases such as Alzheimer's disease (AD).

Oxygen radicals

The formation of oxygen radicals in the brain is achieved through the nitric oxide synthase (NOS) pathway. This reaction occurs as a response to an increase in the Ca2+ concentration inside a brain cell. This interaction between the Ca2+ and NOS results in the formation of the cofactor tetrahydrobiopterin (BH4), which then moves from the plasma membrane into the cytoplasm. As a final step, NOS is dephosphorylated yielding nitric oxide (NO), which accumulates in the brain, increasing its oxidative stress.[6]

There are several ROS including: superoxide, hydrogen peroxide and hydroxyl, all of which lead to neurotoxicity. Naturally, the body utilizes a defensive mechanism to diminish the fatal effects of the reactive species by employing certain enzymes to break down the ROS into small, benign molecules of simple oxygen and water.[7]

However, this breakdown of the ROS is not completely efficient; some reactive residues are left in the brain to accumulate, contributing to neurotoxicity and cell death. The brain is more vulnerable to oxidative stress, in comparison to other organs, due to its low oxidative capacity.

Because neurons are characterized as postmitotic cells, meaning that they live with accumulated damage over the years, accumulation of ROS is fatal. Thus, increased levels of ROS age neurons, which leads to accelerated neurodegenerative processes and ultimately the advancement of AD.[8]

How Antioxidants Work

As cells function normally in the body, they produce damaged molecules called free radicals, which are highly unstable and steal components from other cellular molecules, such as fat, protein, or DNA, thereby spreading the damage. This process, called peroxidation, continues in a chain reaction, and entire cells soon become damaged and die. Peroxidation is important because it helps the body to destroy cells that have outlived their usefulness and kills germs and parasites. However, peroxidation, when left unchecked, also destroys or damages healthy cells.

Antioxidants help prevent widespread cellular destruction by donating components to stabilize free radicals. More important, antioxidants return to the surface of the cell to stabilize, rather than damage, other cellular components.

When there are no enough antioxidants to hold peroxidation in check, free radicals begin damaging healthy cells, which can lead to problems. For example, free radical damage to immune cells can lead to an increased risk of infections.

Flavonoids are low molecular weight bioactive poly phenols[9] which play a vital role in photo synthesizing cells. The original "flavonoid" research apparently began in 1936, when Hungarian scientist Albert Szent-Gyorgi was uncovering a synergy between pure vitamin C and as yet un identified co-factors from the peels of lemons, which he first called "citrin," and later, "vitamin P".[10]

Flavonoids have been reported to exert wide range of biological activities. These include, anti-inflammatory, antibacterial, antiviral, anti-allergic,[10] cytotoxic anti-tumour treatment, neurodegenerative diseases and vasodilator action.[11] In addition flavonoids are known to inhibit lipid-peroxidation, platelet aggregation, capillary permeability and fragility, cyclo-oxygenase and lipoxygenase enzyme activities.

They exert these effects as antioxidants, free radical scavengers, cheaters of divalent cation.[12] These are also reported to inhibit variety of enzymes like hydrolases, hyalouronidase, alkaline phosphatise, aryl sulphates, cAMP phosphodiesterase, lipase, α-glucosidase and kinase.[13]

Diosmin is a naturally occurring flavonoid glycoside, it is a member of the citrus flavonoid family and is a modified form of hesperidin.[14] It is a semisynthetic drug that is used as an oral phlebotropic drug in the treatment of venous disease, i.e., chronic venous insufficiency and haemorrhoidal disease. Diosmin prolong the vasoconstrictor effect of nor epinephrine on the vein wall, increased the venous tone, and therefore reduces venous capacitance, distensibility, and stasis.[14] Diosmin has been found to be effective in mitigating hyperglycaemia in diabetic rats.[15]

Its anti-inflammatory and anti-apoptotic activity has been demonstrated in neuronal cells. Until now, however, clinical studies of the effects of Diosmin have been in conclusive. Furthermore, only a limited number of articles have been published and few articles are available on the use of diosmin. Based on the previous studies I like to carry out the evaluation of neuroprotective activity of Diosmin on Wistar rats.

A) ARSENIC INDUCED NEUROTOXICITY

The word arsenic is derived from the Persian *zarnikh.* As was used in traditional Chinese and Indian medicine and as a cosmetic product in eye shadow in the Roman era. Arsenic is used in folk medicine and in pesticides in many countries and also in modern western medicine for the treatment of leukemia. In traditional Chinese medicine, preparations can be obtained in the form of coated or uncoated pills, powder or syrups.[16]

Arsenic is used in folk medicine and in pesticides in many countries and also in modern western medicine for the treatment of leukemia.[17,18] In traditional Chinese medicine, preparations can be obtained in the form of coated or uncoated pills, powder or syrups. Different studies have shown that the majority of traditional Chinese medicines, such as Chinese herbal balls, show high doses of As varying between 0.1 and 36.6 mg per tablet, causing patients to get intoxicated by the high As dose, and Indian ayurvedic herbal medicine products are also known to cause lead, mercury and As intoxication.[19-22]

Nowadays, the therapeutic use of As is making a comeback in modern medicine. Arsenic trioxide (ATO), for instance, is used to treat patients with relapsed acute promyelocytic leukemia (APL).[23-26] But ATO is also known for its less favorable side, as in causing temporary cardiac and neurotoxic side effects in APL patients.[18]

Arsenic was one of the primary ingredients in pesticides before synthetic organic pesticides were available; its long-term application in agricultural pesticides has resulted in high levels of arsenic in the body of workers, who are exposed by inhalation during the spraying. Arsenic-containing rodent pesticides used for pest and insect control were banned due to human health concerns in production, use, and accidental poisoning and possible abuse in intentional poisoning. Various case reports and studies have revealed that exposure to As has resulted in various forms of cancer and peripheral neuropathy.[17,27, 28]

Acute and chronic exposure to arsenic

Acute:

A single exposure, to a high dose may lead to severe reactions such as diarrhea, vomiting, pain, dehydration and weakness. Nowadays, acute intoxication rarely occurs in western European countries; if it occurs, it is usually the result of intentional (suicide or homicide) or accidental poisoning. Occupational exposure to As is rare and usually occurs in the form of arsine gas, which causes symptoms different to those caused by As ingestion.[29]

Exposure often occurs when arsine gas escapes during transport or when it is generated while arsenic-containing ores or metals are treated with acid.[30,31] Acute oral exposure to As is associated with gastrointestinal symptoms such as nausea, vomiting, abdominal pain and severe diarrhea. Cardiovascular and respiratory symptoms include hypotension, shock, pulmonary edema and heart failure. If survived, acute poisoning with As is also combined with neurological symptoms like light-headedness, weakness, delirium, encephalopathy and peripheral neuropathy, which have been reported.[32]

Peripheral neuropathy as a result of As intoxication may be delayed several weeks after the initial toxic insult.[33] After a few weeks the patients show signs of recovery, however, when tested with electrophysiological studies 6 to 8 years after exposure, the patients still showed reduced motor conduction velocity.[33]

Chronic

Environmental and occupational As exposure is not only caused by contaminated drinking water due to the leaching of natural geological resources, but may also occur from mining and other industrial processes.[34]

Chronic ingestion of low concentration of As levels can occur through industrial accidents, work and environment, which eventually may cause a higher tolerance for As. An example of such an industrial accident can be found in the early 20th century, in reports on As intoxication caused by contaminated beer in Salford, UK. The patients, had a few symptoms in common, they all suffered from 'peripheral neuritis' characterized by weakness in the limbs that made it difficult for them to walk. [35,36]

In several cases some of the patients suffered from rashes and itching, sometimes these complaints were accompanied by darkening of skin texture. The presence of As in the beer was due to the use of contaminated glucose and inverted sugar by the brewers. Furthermore, the contamination of this sugar is caused by the use of very impure sulphuric acid. The source of As contamination was traced back to invert sugar, which was caused by the action of sulphuric acid on various forms of starch. The original source was the Spanish pyrites from which the sulphuric acid was made and often contained large percentages of As.

Chronic As poisoning represents a global, serious health concern, if As can be found in high concentrated levels in the environment from natural or industrial processes. Arsenic contamination of groundwater has occurred in various parts of the world such as in the Americas, Bangladesh, India, Taiwan and many other Asian countries. In Bangladesh, a country of 125 million, between 35-77 million people are currently exposed to As through drinking water, which is also the major cause of death, especially among children.[37,38]

As a quick and inexpensive solution for the lack of sufficient and clean drinking water, UNICEF and the World Bank advocated to tap into deeper groundwater. Millions of wells were constructed; infant mortality and gastrointestinal illness were reduced by half. However, later studies revealed that over 40% of these wells are contaminated with arsenic. As a result, due to the daily exposure to As in their drinking water, the population in south east Asia has to endure various ailments caused by As.

Chronic symptoms of As exposure are pigmentation changes, gastrointestinal symptoms, anemia, liver disease, a metallic taste and Mees' lines on the nails,[39] blackfoot disease and diabetes mellitus.[40] Apart from pigmentation changes, arsenic-induced skin pathology caused by chronic intoxication also causes hyperkeratosis, Bowen's disease, squamous cell carcinoma and basal cell carcinomas.[41,42]

Hafemann et al. (2005) have shown an association between arsenic exposure and peripheral neuropathy in the Bangladesh population that has been chronically exposed to arsenic in drinking water. They showed that increased As exposure, as measured by both cumulative and urinary measures, was associated with evidence of sub-clinical sensory neuropathy. The correlation between As exposure and neuropathy was shown with the increased vibrotactile threshold, a sign of subclinical sensory neuropathy and sub-clinical loss of vibratory sensation in the lower extremities.[43]

Kinetics of various arsenic metabolites and their molecular mechanism of toxicity

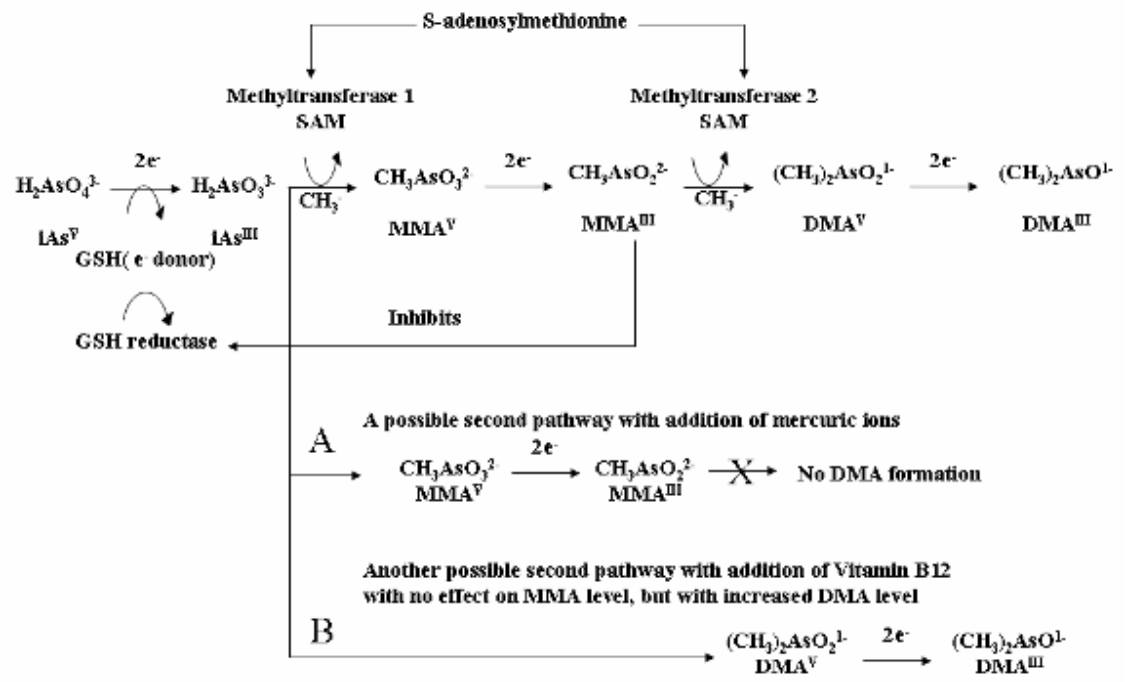
Kinetics.

Arsenic absorption takes place mainly in the small intestines; also a minimal absorption occurs from skin contact and inhalation.[41,44,45] After ingestion, As is metabolized from inorganic to organic compounds with varying degrees of toxicity and the behavior of the different As species vary markedly. Arsenic speciation of inorganic and organic forms of As is often as important as total quantification, because of their different toxicity and mobility.[46]

As speciation in biological samples is an essential tool to gain insight into its distribution in tissues and its specific toxicity to target organs. Arsenic metabolites exist both in organic and inorganic forms and both types can exist in either trivalent or pentavalent oxidation states. The bioavailability of inorganic As is up to 60%. Inorganic As such as arsenate (iAsV), the pentavalent form, and arsenite (iAsIII), the trivalent form, are the most aggressive single-substance toxicants, specially the trivalent form.

A redox reaction reduces the pentavalent As to its trivalent state. This reduction step from pentavalent to trivalent, releases a more toxic compound, which in fact results in bioactivation.

Fig. 1: Metabolic pathways of arsenate methylation



Above mentioned figure explains the two possible pathways for arsenate methylation. The main pathway is a straightforward bioactivation (metabolic activation) of arsenate in MMA, DMA and the theoretical conversion into TMA (not shown).[47,48,49] A possible second pathway is direct metabolism of iAs (A) to either MMA without further conversion to DMA or (B) increase in DMA levels, while the MMA levels stay intact.[50]

Arsenic metabolism shows a common route of absorption, distribution and excretion both in humans and various animals with subtle interspecies differences. Metabolism of inorganic As starts with intake and absorption. Distribution of As varies per species. This is a result of various factors such as species, bodyweight, route of intake and duration of exposure. After its absorption, As can be found in different organs, especially in the liver.[50,52]

Arsenic undergoes hepatic biomethylation to form monomethyl arsenic (MMA) and dimethyl arsenic (DMA). Detoxification starts with transformation of inorganic As to organic As and reduction of pentavalent arsenate to trivalent arsenite, which results in a more toxic component. The reduction of arsenate to arsenite is catalyzed by gluthatione (GSH) and other thiols, which are reducing agents.[45] Arsenate at physiological pH is ionized and as such is not able to pass cellular membranes. Conversion to arsenite at physiological pH facilitates passage through the cellular membrane.

Metabolism of As continues by using arsenite as its substrate for methylation, which is distributed in tissues and cytosol. However, methylation does not appear to be the primary detoxification pathway for arsenite. Protein binding has been suggested as the initial protective mechanism. Arsenite binding to protein serves as a reservoir and takes place after initial increase in arsenite concentration. When methylation enzymes start to become effective, the reservoir may slowly release small amounts of arsenite for methylation.[52,53]

The methylation of arsenite is catalyzed by a specific methyltransferase using S-Adenosyl methionine as a methyl donating cofactor (SAM).[43,47] Addition of a methyl group to arsenite leads to synthesis of pentavalent monomethylarsonic (MMAV). The MMAV in turn is reduced to trivalent MMA (MMAIII) by GSH, the reducing agent. Another round of methyltransferase activity with MMAIII results in production of dimethylarsenic acid (DMA).

However, it is not clear whether the same SAM is used for methylation of MMA and DMA or that two separate SAMs are being used for these two methylation steps. Theoretically, conversion of arsenate to DMA could be extended by one more round to achieve trimethylarsenic acid (TMA).[52,54] A second possible conversion route to MMA and DMA was introduced by the in vitro studies of Buchet and Lauwerys, by adding mercuric ions; they prevented the formation of DMA without affecting MMA formation.[50,51]

Furthermore, it was evident that the addition of cyanocobalamin or methyl-cyanocobalamin (vitamin B12) and coenzyme B12 in combination with SAM resulted in a significant increase of DMA and no additional increase in MMA level. This experiment revealed two different enzymatic activities involved in methytransferase to either MMA or DMA.

The methylation process has been thought to be the detoxification mechanism for As. This is true when this line of reasoning is applied to parameters of acute toxicity of As species, such as LC50 or LD50. As a rule, the trivalent As compounds are more cytotoxic then their pentavalent forms. However, various studies in animals and cell cultures have shown the adverse effects of methylated As, such as DMAV as a tumor promoter [55,56] or direct genotoxic action of MMAIII and DMAIII in vitro.[52]

The main excretion route of As is through the urine and bile. However, the various As metabolites do not excrete in the same fashion in different animals and humans. For example, studies in rats have shown that pentavalent metabolites such as iAsV, MMAV and DMAV are

exclusively excreted into urine, MMAIII only into bile and iAsIII into both bile and urine.[57] In contrast, human studies revealed the presence of MMAIII in urine.[48,58] Arsenic studies carried out by Csanaky and Gregus in rats, mice, hamsters, rabbits and guinea pigs revealed some similarities. All species injected with iAsV excreted various As metabolites into urine, in contrast to injection with iAsIII, which showed higher excretion into bile rather than urine.[57]

Mechanism

These metabolites exert their toxicity by inactivating many enzymes, especially those involved in the cellular energy pathway and DNA synthesis and repair. Arsenic is substituted for phosphate in high-energy compounds such as ATP. Arsenic binds covalently with sulfhydryl groups in their reduced form. These interactions also take place with certain enzymes necessary for cellular metabolism.[27]

Various As carcinogenesis studies have revealed that As may alter one or more DNA repair processes. Andrew et al. have shown that patients exposed to As have altered nucleotide excision repair mainly on the excision repair cross-complementing 1 (ERCC1) component. Arsenic exposure was associated with decreased expression of ERCC1 in isolated lymphocytes at the mRNA and protein levels.[59]

In short, As exposure results not only in general toxicity but also in neuronal diseases and carcinogenesis. Tri-valent arsenic (iAsIII, MMAIII and DMAIII) compounds are thought to interact with thiol groups of proteins and enzymes in their reduced state. This is believed to inhibit the catalytic activity of enzymes.[58] The common thinking is that tri-valent arsenic metabolites inhibit pyruvate dehydrogenase (PDH), which leads to disruption of the energy system of the cell,[59] which in turn may release an apoptosis-inducing factor (AIF) resulting in cell damage and death.

AIF is released from the mitochondrial intermembrane space from where it translocates to the cell nucleus.[60] Apoptosis is associated with early formation of ring-like perinuclear condensed chromatin co-localized with AIF, DNA fragmentation and finally cell death. Pentavalent As (iAsV, MMAV and DMAV) is substituted for phosphorus in many biochemical reactions. Replacing the stable phosphorus anion in phosphate with the less stable AsV anion leads to rapid hydrolysis of high-energy bonds in compounds such as ATP. At the level of the citric acid cycle, As inhibits succinate dehydrogenase and by competing with phosphate it

uncouples oxidative phosphorylation, thus inhibiting energy-linked reduction of NAD+, mitochondrial respiration, and ATP synthesis. This leads to loss of high-energy phosphate bonds and effectively uncouples oxidative phosphorylation.[61,49]

Another approach is that trivalent As inhibits enzyme complexes by reactive oxygen species (ROS), indicating that ROS production by trivalent As causes pyruvate dehydrogenase (PDH) inactivation through oxidation.[62] Inactivation through oxidation causes impaired gluconeogenesis and reduced oxidative phosphorylation.[63] Production of ROS by As was determined by use of a nonfluorescent dye 5',6'-chlomethyl-2',7'-dichlorodihydrofluorscein (CM-H2DCFDA), which is a non-specific radical detector to identify the radical species.[64]

Pharmacological and Toxicological Effects of Arsenic [65]

Arsenicals have varied effects on many organ systems, as summarized below.

Cardiovascular System

Acute and subacute doses of inorganic arsenic induce mild vasodilation. Serious cardiovascular effects include hypotension, congestive heart failure, and cardiac arrhythmias. Long-term exposure results in peripheral vascular disease, more specifically gangrene of the extremities, especially of the feet, often referred to as blackfoot disease. Myocardial damage and hypotension may become evident after more prolonged exposure to arsenic.

Gastrointestinal Tract

Acute or subacute exposure to arsenic can produce GI disturbances, with chronic exposure to arsenic, GI effects usually are not observed. Small doses of inorganic arsenicals, especially the trivalent compounds, cause mild splanchnic hyperemia. Normal proliferation of the epithelium is suppressed, which accentuates the damage. Soon the feces become bloody. Damage to the upper GI tract usually results in hematemesis. Stomatitis also may be evident. The onset of GI symptoms may be so gradual that the possibility of arsenic poisoning may be overlooked.

Kidneys

The action of arsenic on the renal capillaries, tubules, and glomeruli may cause severe renal damage. Initially, the glomeruli are affected, and proteinuria results. Varying degrees of tubular necrosis and degeneration occur later. Oliguria with proteinuria, hematuria, and casts frequently results from arsenic exposure.

Skin

Skin is a major target organ of arsenic. Diffuse or spotted hyperpigmentation over the trunk and extremities usually is the first effect observed with chronic arsenic ingestion. Long-term ingestion of low doses of inorganic arsenicals causes cutaneous vasodilation and a "milk and roses" complexion. Eventually, skin cancer is observed.

Blood

Inorganic arsenicals affect the bone marrow and alter the cellular composition of the blood. Hematological evaluation usually reveals anemia with slight-to-moderate leukopenia; eosinophilia also may be present. The vascularity of the bone marrow is increased.

Liver

Inorganic arsenicals and a number of now-obsolete organic arsenicals are particularly toxic to the liver and produce fatty infiltration, central necrosis, and cirrhosis.

Carcinogenesis

The association of arsenic exposure and skin tumors was noted more than 100 years ago in patients treated with arsenicals. The International Agency for Research on Cancer concluded that inorganic arsenic is a skin and lung (via inhalation) carcinogen. Increased risks of other cancers, such as kidney and liver cancer, also have been reported, but the association with arsenic is not as high as for the tumors just noted.

Nervous System

High-dose acute or subacute exposure to arsenic can cause encephalopathy; however, the most common arsenic-induced neurological lesion is a peripheral neuropathy with a stocking/glove distribution of dysesthesia. This is followed by muscular weakness in the

extremities, and with continued exposure, deep-tendon reflexes diminish, and muscular atrophy follows. The cerebral lesions are mainly vascular in origin and occur in both the gray and white matter; characteristic multiple symmetrical foci of hemorrhagic necrosis occur.

Arsenic-induced neurotoxicity

Arsenic effects manifest themselves weeks after first exposure as both central and peripheral neuropathy. Central neuropathy due to As poisoning has been reported to cause impairment to neurological functions such as learning, short-term memory and concentration.[65] People chronically poisoned by arsenic occurring naturally in groundwater may suffer from toxic delirium and encephalopathy.[66] Neuropsychological tests showed mildly impaired psychomotor speed and attentive processes, whereas verbal learning and memory were severely impaired.

The most frequent neurological manifestation by As is peripheral neuropathy that may last for several years or even life-long. The peripheral neuropathy may lead to rapid severe ascending weakness, similar to the Guillan-Barré syndrome, requiring mechanical ventilation. Peripheral neuropathy is common in persons chronically exposed to As-contaminated drinking water.[65]

From human clinical cases studied by Le Quesne and McLeod it has become clear that As exposure results in a latent reaction to the nervous system, which was established through their reduced Nerve Conduction Velocities (NCVs) measurements.[33] These patients showed some recovery in the years following exposure to As; however, a full NCV regain was not achieved. It is doubtful whether PNS symptoms will ever disappear completely. Patients exposed to As show significantly lower NCVs in their peripheral nerves incomparison to their referents.[32,67,48]

Perhaps, changes in cytoskeletal composition may be the major reason of As poisoning leading to axonal degeneration, which in turn could lead to axonopathy. An actual mechanistic model for arsenic neurotoxicity is as yet not easy to hypothesize, although interference with cytoskeletal proteins is a primary consideration. Therefore, one may look for such mechanistic parallels in other neurotoxins, especially neurotoxic metals, but also in other neurodegenerative diseases. Metals as environmental pollutants such as lead and mercury have been associated with Neurodegenerative diseases[69]

Exposure to aluminum, lead and mercury are known to have caused abnormalities in the nervous system related to interference with the cytoskeleton. Clinically, symptoms may occur as peripheral and central neuropathies. Aluminum has been known to cause dialysis encephalopathy in some individuals with renal failure. Furthermore, it has been suggested that aluminum might be implicated in Alzheimer's disease (AD), because of some similarities in pathological changes. The pathological changes in both cases can be summarized as presence of neurofibrillary tangles as diagnostic hallmarks AD.[70]

However, further examination of these tangles has shown differences in their tangles between AD and the aluminum-exposed patients, in AD patients tangles consist of paired helical filaments, whereas those induced by aluminum are single.[71] Aluminum exposure in animal studies has shown induction of neurofibrillary degeneration. Phosphorylation of cytoskeletal proteins appears to modulate their interactions with one another and with other cellular proteins. Disruption of the phosphorylation of cytoskeletal proteins results in disorganization of the cytoskeletal structure.[72]

Other metals as neurotoxicants such as lead have also been indicated in the etiology of amyotrophic lateral sclerosis (ALS), whereas manganese has been reported to be involved in Parkinson's disease (PD) or a similar syndrome, Parkinsonism. Exposure to lead has been shown to be related to ALS in a case control study conducted in New England from 1993 to 1996.[73,74] Generally, ALS is divided into two forms: (1) the classic sporadic form; (2) the familial, presumably hereditary form. The cause of the sporadic form is unknown. The cause of the familial form is believed to be genetic, attributable to a mutation in Cu–Zn superoxide dismutase.[75]

Studies in occupational exposure have suggested an association between PD and elevated exposure to manganese.[76] Exposure to manganese can cause neurotoxicity and a neurological syndrome that resembles PD.[77] Neurofilament proteins are major constitutes of neurons and they control axonal caliber, transport and signal.[78] In neurodegenerative diseases such as AD it appears that the metabolism of neurofilaments is disturbed, as indicated by the presence of neurofilament epitopes in the neurofibrillary tangles, as well as by the severe reduction of the expression of the gene for the light neurofilament subunit of the neurofilament triplet (neurofilament High, - Middle and –Light) in brains of AD patients.[79]

Accumulation of neurofilaments in the proximal cell body and the perikaryon of motor neurons is a hallmark of ALS and PD.[75,78] Disruption and disorganization of neurofilament transport and neuron cytoskeletal network is a pathological feature seen in all of these neurodegenerative diseases. In rats exposed to As , decrease of the neurofilament Light subunit (NF-L) in sciatic nerve is evident.[80] Arsenic-induced decrease of NF-L may play an important role in the pathological changes of the nervous system, since NF-L is the only NF protein capable of independently organizing and co-assembling filaments in vivo. Both NF-H and NF-M need NF-L protein to form a heteropolymer in the cytoskeletal framework.[81]

However, in vitro studies with iAsIII in neuroblastoma (SK-N-SH) and Schwannoma (ST-8814) cell lines show no effect on their mRNA expression level of cytoskeletal genes.[82] Thus, it can be suggested that the decrease in NF-L expression is a post-translational activity as a result of a proteolytic process. Calpain (calcium-activated cytoplasmic protease) could be responsible for NF-L degradation, since neuroblastoma cells (SY-5Y) treated with arsenic trioxide (trivalent As) show an increase in intracellular calcium.[83]

Studies in PC12 cells under oxidative stress circumstances have shown an increase of calcium in the cells and up-regulation of calpain leading to degradation of NF-L protein.[84] Furthermore, inactivation of calpain by calpain inhibitor (MDL-28170) prevents NF-L breakdown.[85,86] These results suggest that As-induced destabilization and disruption of the cytoskeletal framework is partly due to activation of calpain, through influx of Ca2+, which in turn is responsible for NF-L degradation in a calcium-induced proteolytic process.

Another important cytoskeletal protein in neurodegenerative diseases is the tau protein (MAPtau), which is a member of the microtubule protein family transcripted by alternative splicing of a single gene. It has tandem repeats of a tubulin binding domain and promotes tubulin assembly. Although tau proteins are found in all cells, they are major components of neurons where they are predominantly associated with microtubules of the axon. Changes in tauprotein may play a role in the pathogenesis of neurodegenerative diseases.

In AD patients, MAP-tau becomes abnormally hyperphosphorylated and accumulates as tangles of paired helical filaments in neurons undergoing degeneration. Hyperphosphorylated MAP-tau disorganizes microtubules assembly from normal tau and tubulin, which may lead to the formation of the neurofibrillary tangles and the degeneration of the affected neurons in AD

patients. Arsenic may affect the phosphorylation of tau-proteins as well. Giasson et al. demonstrated hyperphosphorylation of tau-proteins in Chinese hamster ovary (CHO) cells in vitro after treatment with iAsIII.[87]

They also showed that iAsIII causes a significant increase in the phosphorylation of several amino acid residues in tau. This is in line with experiments in rats with iAsIII, which have also shown that the rats' MAP-tau was hyperphosphorylated after dosing them with iAsIII {unpublished data}. These results indicate that As may be involved in the cascade leading to deregulation of tau function associated with neurodegeneration.

Diagnosis and treatment of Arsenic (As) poisoning

Arsenic concentration measurements for diagnostic purpose are usually carried out in urine. Acute As toxicity is usually diagnosed by increased urinary As in excess of 50 cg/l urine sample or 100 cg in 24-hour urine, and a shorter time span before examination, if no seafood has been ingested. The urine is collected in metal-free containers. Other biological samples, such as blood, and even hair and nails in chronic cases, are also used in the clinical laboratory. For treatment of acute As poisoning, the primary concern is to correct the dehydration caused by As and restore vital bodily functions.

In order to increase elimination, physicians prescribe gastric lavage and activated charcoal, but haemodialysis may also be considered. The efficacy of these detoxification methods, however, has not been well recorded. Although the metalloid As cannot be categorized as a metal, it shows some metal properties. Based on this fact, chelators can be used to remove As ions from the body. For treatment of acute As poisoning, the chelator 2.3-dimercapto-1-propanol (British Anti-lewisite, BAL) has been used with successful results.[88,67]

Patients who were administered this drug showed elevated As excretion in their urine. At follow-up, their urinary As concentration was decreased to the background level. At the moment of admittance, neurological examination demonstrated no signs of nervous system depression. However, these patients survived the high dose of ingested As with only latent neuropathy symptoms. Neurological complications such as distal, symmetrical, sensory, axonal neuropathy

are late effects of acute As poisoning. These neurological effects are non-responsive to chelation.[89]

In clinical cases with chronically poisoned patients, trials with 4 As chelators such as BAL, meso-2.3-dimercaptosuccinic acid (DMSA), D-penicillamine and sodium 2.3-dimercapto-1-propamesulfonate (DMPS) did not provide any clinical, biochemical or histopathological benefits.[68,9092] On the other hand studies done in rats with an As chelator such as BAL showed depletion of tissue As and its excretion via urine and faeces.[38] Although the binding affinity of a chelating agent for the metal is greater than for endogenous ligands, chelating is generally ineffective for treating established arsenical peripheral neuropathy.[39]

After long-term exposure to arsenic, treatment with dimercaprol and penicillamine also may be used, but oral penicillamine alone usually is sufficient. The duration of therapy is determined by the clinical condition of the patient, and the decision is aided by periodic determinations of urinary arsenic concentrations. Dialysis may become necessary with severe arsenic-induced nephropathy; successful removal of arsenic by dialysis has been reported (Vaziri et al., 1980).

Antioxidants are seemingly magical nutrients that can repair cell damage that happens in all our bodies over time, including those of our cats. These nutrients occur naturally, common antioxidants include vitamin A, vitamin C, vitamin E and certain compounds called carotenoids (like lutein and beta-carotene).

B) IMPORTANCE OF FLAVONOIDS IN OXIDATIVE STRESS

Flavonoids belong to a group of natural substances with variable phenolic structures and are found in most plant species, and account for a significant percentage of the chemical constituents of some; e.g. dried green tea leaves contain approximately 30% flavonoids by weight.

Flavonoids have been shown the very good antioxidant activity of flavonoids-their ability to scavenge hydroxyl radicals, superoxide anions, and lipid peroxy radicals-may be the most important function of flavonoids, and underlie many of the above actions in the body.

Oxidative damage is implicated in most disease processes, and epidemiological, clinical, and laboratory research on flavonoids and other antioxidants suggest their use in the prevention and treatment of a number of diseases.

These natural products were known for their beneficial effects on health long before flavonoids were isolated as the effective compounds. More than 4000 varieties of flavonoids have been identified.[94]

Flavonoids, or bioflavonoids, are a ubiquitous group of polyphenolic substances which are present in most plants, concentrating in seeds, fruit skin or peel, bark, and flowers. A great number of plant medicines contain flavonoids, which have been reported by many authors as having antibacterial, anti-inflammatory, anti-allergic, anti-mutagenic, antiviral, antineoplastic, anti-thrombotic, and vasodilator actions.

The structural components common to these molecules include two benzene rings on either side of a 3-carbon ring.

Multiple combinations of hydroxyl groups, sugars, oxygen's and methyl groups attached to these structures create the various classes of flavonoids.[95]

Classification of flavonoids and their common sources [96,97]

Chemical Class	**Examples**	**Major Dietary Source**
Flavonols	Quercetin, Rutin, Myricetin, Kaempherol	Tea, Red wine, Apple, Tomato, Cherry and Onion
Flavanols	**Monomers** (Catechins): Catechin, Epicatechin, Epigallocatechin Epicatechin gallate, Epigallocatechin. **Dimers and Polymers**: Theaflavins, Thearubigins, Proanthocyanidins	**Catechins**: Tea, chocolate, grapes, berries, apples **Theaflavins, Thearubigins**: Tea **Proanthocyanidins:** Chocolate, apples, berries, red wine
Flavones	Apigenin, Chrysin, Luteolin	Thyme and Parsley
Isoflavones	Genistein, Glycitein, Daidzein, Formononetin.	Soya bean and other legumes
Flavanones	Hesperidin, Narigenin	Grapefruit and Orange
Flavanonols	Taxifolin	Lemon and Sour orange
Anthocyanidins	Cyanidin, Delphinidin, Malvidin, Pelargonidin, Peonidin, Petunidin	Red, blue, and purple berries; red and purple grapes; red wine

Pharmacological effects of Flavonoids

Antioxidant effect [95]

Flavonoids have been shown in a number of studies to be very good antioxidants, capable of scavenging hydroxyl radicals, superoxide anions, and lipidperoxy radicals. Free radicals, including the superoxide radical ($O2^{\cdot}$-), hydroxyl radical (.OH), hydrogen peroxide (H2O2) , and

lipid peroxide radicals have been implicated in a number of disease processes, including asthma, cancer, cardiovascular disease, cataracts, diabetes, gastrointestinal inflammatory diseases, liver disease, macular degeneration, periodontal disease, and other inflammatory processes.

These radical oxygen species (ROS) are produced as a normal consequence of biochemical processes in the body and as a result of increased exposure to environmental and/or dietary xenobiotics. ROS are also beneficial components of the immune response, hepatic cytochrome P450-mediated detoxification, and regulation of smooth muscle tone. It is an imbalance in the oxidant versus antioxidant processes (oxidative stress) that is thought to cause the subsequent cellular damage which leads to the disease processes named above.

The body's antioxidant systems, including superoxide dismutase, catalase, and glutathione, should keep the oxidative processes in check; however, deficiencies of nutritional antioxidants (flavonoids; vitamins A, C, E; the minerals selenium and zinc; coenzyme Q10, lipoic acid; and L-cysteine), and/or an over whelming oxidant stress can overload this system.16 The mechanism of free-radical damage includes ROS-induced peroxidation of polyunsaturated fatty acids in the cell membrane lipid bilayer, which causes a chain reaction of lipid peroxidation, thus damaging the cellular membrane and causing further oxidation of membrane lipids and proteins. Subsequently, cell contents, including DNA, are damaged. It is this free radical-induced damage which is thought to precede these overt disease processes.

Anti-atherosclerotic effects [98]

Because of their anti-oxidative properties, flavonoids are likely to have a major influence on the vascular system. Oxygen radicals can oxidize LDLs, which injures the endothelial wall and there by promotes atherosclerotic changes. A few clinical studies have pointed out that flavonoid intake protects against coronary heart disease, the flavonoids in regularly consumed foods might reduce the risk of death from coronary heart disease in elderly men. Oxidative stress and vascular damage are postulated to play a key role in dementia; the intake of flavonoids appears inversely related to the risk of dementia.

Anti thrombogenic effects [95]

Platelet aggregation contributes to both the development of atherosclerosis and acute platelet thrombus formation, followed by embolization of stenosed arteries. Activated platelets

adhering to vascular endothelium generate lipid peroxides and oxygen free radicals, which inhibit the endothelial formation of prostacyclin and nitrous oxide. Selected flavonoids, such as quercetin, kaempferol, and myricetin were shown to be effective inhibitors of platelet aggregation.

Flavonols are particularly antithrombotic because they directly scavenge free radicals, thereby maintaining proper concentrations of endothelial prostacyclin and nitric oxide. Flavonoids are powerful antithrombotic agents in vitro and in vivo because of their inhibition of the activity of cyclooxygenase and lipoxygenase pathways. It is well known that arachidonic acid, which is released in inflammatory conditions, is metabolized by platelets to form prostaglandin, endoperoxides, and thromboxane A2, leading to platelet activation and aggregation.

The main anti aggregatory effect of flavonoids is thought to be by inhibition of thromboxane A2 formation. Flavonoids affect arachidonic acid metabolism in different ways. Some flavonoids specifically block cyclooxygenase or lipoxygenase, whereas others block both enzymes. In vitro studies showed that flavonoids bind to platelet membranes and may therefore have an accumulative effect over time.

Anti-tumour effects [96]

Antioxidant systems are frequently inadequate, and damage from reactive oxygen species is proposed to be involved in carcinogenesis. Reactive oxygen species can damage DNA, and the division of cells with unrepaired or misrepaired damage leads to mutations. Reactive oxygen species can interfere directly with cell signalling and growth. The cellular damage caused by reactive oxygen species can induce mitosis, increasing the risk that damaged DNA will lead to mutations, and can increase the exposure of DNA to mutagens. It has been stated that flavonoids, such as antioxidants, can inhibit carcinogenesis. Some flavonoids such as apigenin, fisetin and luteolin are stated to be very good inhibitors of cell proliferation.

Anti-inflammatory effects

Cyclooxygenase and lipoxygenase play an important role as inflammatory mediators. They are involved in the release of arachidonic acid, which is a starting point for a general inflammatory response. Selected phenolic compounds were shown to inhibit the cyclooxygenase

and 5-lipoxygenase pathways. This inhibition reduces the release of arachidonic acid. Quercetin inhibits both cyclooxygenase and lipoxygenase activity, thus diminishing the formation of these inflammatory metabolites.

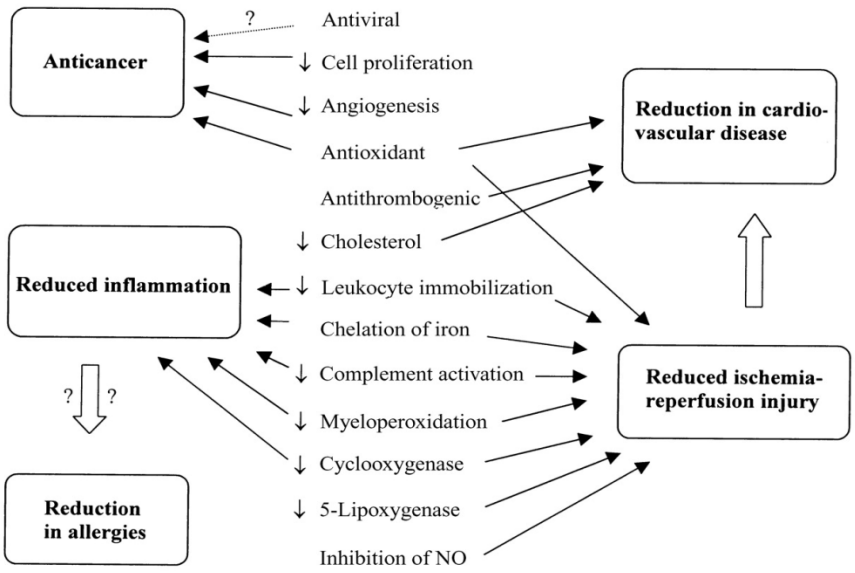
Another anti-inflammatory feature is that flavonoids also inhibit eicosanoid biosynthesis. Eicosanoids, such as prostaglandins, are involved in various immunologic responses. Flavonoids also inhibit both cytosolic and membrane tyrosine kinase. Integral membrane proteins, such as tyrosine 3-monooxygenase kinase, are involved in a variety of functions, such as enzyme catalysis, transport across membranes, and transduction of signals that function as receptors of hormones and growth factors, and energy transfer in ATP synthesis.

Inhibition of these proteins results in inhibition of uncontrolled cell growth and proliferation. Tyrosine kinase substrates seem to play key roles in the signal transduction pathway that regulates cell proliferation. Another anti-inflammatory property of flavonoids is their suggested ability to inhibit neutrophil degranulation. This is a direct way to diminish the release of arachidonic acid by neutrophils and other immune cells.

Flavonoids inhibit the angiogenesis, which is regulated by a variety of endogenous angiogenic and angiostatic factors. Pathologic, unregulated angiogenesis occurs in cancer. Angiogenesis inhibitors can interfere with various steps in angiogenesis, such as the proliferation and migration of endothelial cells and lumen formation. Flavonoids seem to play an important role among the known angiogenesis inhibitors.

However, the mechanism behind the antiangiogenic effect of flavonoids is unclear. A possible mechanism could be the inhibition of protein kinase these enzymes are implicated to play an important role in signal transduction and are known for their effects on angiogenesis. Very goodial beneficial effects have been elucidated. The flavonoids as natural compounds have several great advantages over other therapeutic agents because many diets are rich in polyphenolic compounds and are consumed daily having a relatively long half-life with minimum side effects.

Fig. 2. Effect of flavonoids in various diseases[99]



Effect of flavonoids on diabetes mellitus: [100]

Flavonoids can ameliorate some of the consequences of diabetes mellitus. Flavonoids have been identified to be good inhibitors of aldoser-eductase. Effect of flavonoids on diabetes mellitus It has been reported by several researchers that quercetin possess anti-diabetic activity and it has been found that it brings about regeneration of pancreatic islets and increased insulin release in streptozotocin-induced diabetes. It has been reported to stimulate Ca2+ uptake from isolated islet cells thus suggesting it to be effective even in non-insulin dependent diabetes.

Citrus flavonoids:[101]

Citrus fruits are well known for providing ample amounts of vitamin C. But they also supply bioflavonoids; substances that are not required for life but that may improve health. The major bioflavonoids found in citrus fruits are, Diosmin, Hesperidin, Rutin, Naringin, Tangereti, Diosmeti, Narirutin, Neohesperidin, nobiletin and Quercetin.

Citrus bioflavonoids and related substances are widely used in Europe to treat diseases of the blood vessels and lymph system, including hemorrhoids, chronic venous insufficiency, leg

ulcers, easy bruising, nosebleeds, and lymphedema following breast cancer surgery. These compounds are thought to work by strengthening the walls of blood vessels.

Bioflavonoids are also often said to act as antioxidants. Lemon juice has a high antioxidant capacity due to the presence of citrate, vitamin C, vitamin E and flavonoids such as eriocitrin, hesperetin[102] and limonoid.[103] Vitamin E may prevent calcium oxalate crystal deposition in the kidney by preventing hyperoxaluria-induced peroxidative damage to the renal tubular membrane surface (lipid peroxidation), which in turn can prevent calcium oxalate crystal attachment and subsequent development of kidney stones the present compound.[104]

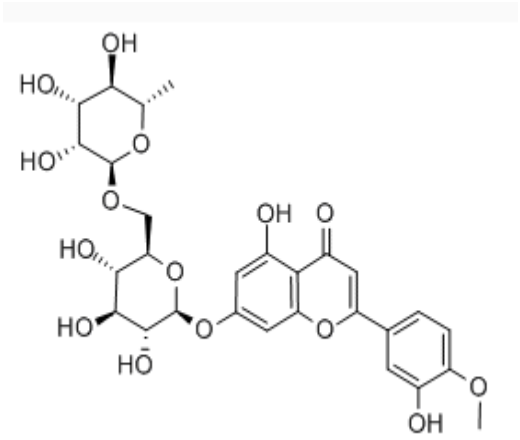
Diosmin is a type of citrus bioflavonoid mostly found in citrus fruits often used as a dietary supplement. Diosmin is considered to be a vascular-protecting agent used to treat chronic venous insufficiency, haemorrhoids, lymphedema, and varicose veins. As a flavonoid, diosmin also exhibits anti-inflammatory, free-radical scavenging, and anti-mutagenic properties.[105]

Science the previous studies reported citrus flavonoids having anti urolithiatic property, antioxidant activity and the present compound is also a citrus flavonoid, we evaluated the effect of diosmin anti urolithiatic activity against the ethylene glycol induced Urolithiasis and nephroprotective activity against gentamicin induced nephron toxicity on Swiss Albino Wistar rats.

2. DRUG PROFILE

Name of the compound: Diosmin

Molecular structure of Diosmin:[106]



Chemical name of Diosmin

{D,(5-Hydroxy-2-(3-hydroxy-4-methoxyphenyl)-7[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-[(2R,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl] oxymethyl] oxan-2-yl]oxychromen-4 one)}

Molecular formula	:	$C_{28}H_{32}O_{15}$
Molecular weight	:	608.54
Melting point	:	278-270°C
Storage temperature	:	2-8°C
Physical Appearance	:	Light yellow to brown yellow powder.
Solubility	:	Insoluble in water and alcohol, Dissolves in dilute alkali hydroxides, and DMSO (50mg/ml).

Description

Diosmin is greyish-yellow or light yellow hygroscopic powder, practically insoluble in water, soluble in dimethyl sulphoxide, practically insoluble in alcohol. It dissolves in dilute solutions of alkali hydroxides.

Dosage

The standard dose of diosmin is 500 mg/day maximum recommended duration of usage was 3 months. Diosmin is a semisynthetic phlebotropic drug, a member of the flavonoid family, a naturally occurring flavone glycoside, used in the treatment of venous disease, i.e., chronic venous insufficiency (CVI) and hemorrhoidal disease (HD), in acute or chronic hemorrhoids, in place of rubber-band ligation, in combination with fiber supplement, or as an adjuvant therapy to haemorrhoidectomy, in order to reduce secondary bleeding. Reduce capillary permeability and to have anti-inflammatory action were collectively known as vitamin P, but these substances, however, are not vitamins.

Diosmin was first isolated in 1925 from Scrophularia nodosa, and first introduced as a therapeutic agent in 1969. Diosmin differs molecularly from hesperidin by the presence of a double bond between two carbon atoms in diosmin central carbon ring. Diosmin can be manufactured by extracting hesperidin from citrus rinds, followed by conversion of hesperidin to diosmin. Diosmin has been used for more than 30 years as a phlebotonic and vascular-protecting agent, and has recently begun to be investigated for other therapeutic purposes, including cancer, premenstrual syndrome, colitis, and diabetes.[105]

Biochemistry and Pharmacokinetics

Flavonoids are a large group of plant pigments sharing the same basic chemical structure; i.e., a three-ringed molecule with hydroxyl (OH) groups attached. Diosmin ($C_{28}H_{32}O_{15}$) occurs naturally as a glycoside, meaning it has a sugar molecule attached to its three-ringed flavonoid structure.

Pharmacokinetic investigations have shown diosmin is rapidly transformed by intestinal flora to its aglycone form, diosmetin. Diosmetin is absorbed and rapidly distributed throughout the body with a plasma half-life of 26-43 hours. Diosmetin is degraded to phenolic acids or their glycine-conjugated derivatives and eliminated through the urine. Diosmin or diosmetin not absorbed is eliminated in the feces.[107,108]

Clinical Indications

Varicose Veins/Chronic Venous Insufficiency

Chronic venous insufficiency is characterized by pain, leg heaviness, a sensation of swelling, and cramps, and is correlated with varicose veins. Diosmin-containing flavonoid mixtures have also been effective in treating severe stages of chronic venous insufficiency, including venous ulceration and delayed healing.[109]

Cancer

Diosmin has been investigated in a number of animal models and human cancer cell lines, and has been found to be chemo preventive andantiproliferative.[110]

Lymphedema

Diosmin acts on the lymphatic system by increasing lymph flow and lymph oncotic pressure. Flavonoid mixture containing diosmin was used to treat upper limb lymphedema secondary to conventional therapy for breast cancer. Results showed improvement of symptoms and limb volume; the mean decrease in volume of the swollen limb reached 6.8 percent. In addition, lymphatic functional parameters assessed with scintigraphy were significantly improved. Animal studies of high-protein lymphedema, such as in burns and lung contusions, showed significant improvement with diosmin.[111]

Diabetes

Diosmin has been shown to improve factors associated with diabetic complications. Blood parameters of glycation and oxidative stress were measured in type 1 diabetic patients before and after intervention with a diosmin-containing flavonoid mixture. A decrease in haemoglobin A1C was accompanied by an increase in glutathione peroxidase, 18 demonstrating long-term decreased blood glucose levels and increased antioxidant activity.

Diosmin can normalize capillary filtration rate and prevent ischemia in diabetics. Rheological studies of type 1 diabetics show diosmin can facilitate hemorheological improvements due to decreased RBC aggregation, which decreased blood flow resistance, resulting in reduction of both stasis and ischemia.[112]

Haemorrhoids

Several large clinical trials have demonstrated diosmin to be effective in the treatment of acute and chronic symptoms of hemorrhoids.

Other clinical indications

Studies have also investigated the use of diosmin for stasis dermatitis, wound healing, premenstrual syndrome, mastodynia, dermato fibro sclerosis, viral infections, and colitis. More clinically oriented research is indicated.[105]

Drug - Nutrient Interactions

Diosmin can cause a decrease in RBC aggregation and blood viscosity. There are no documented cases of adverse interactions between diosmin and prescription medications, but caution should be taken when combining diosmin with aspirin or other blood-thinning medications. Data suggest that diosmin has an inhibitory effect on cytochrome P450 mediated metabolism in healthy volunteers, which may alter the pharmacokinetics of drugs taken concomitantly.

Side Effects and Toxicity

In animal studies, a flavonoid mixture containing 90 percent diosmin and 10 percent hesperidin had an LD50 of more than 3g/kg. In addition, animal studies have shown the absence of acute, sub-acute, or chronic toxicity after repeated oral dosing for 13 and 26 weeks using a dose representing 35 times the recommended daily dose. Diosmin is considered to have no mutagenic activity, embryo toxicity, nor any significant effect on reproductive function. Tran's placental migration and passage into breast milk are minimal.[113]

3. AIM AND OBJECTIVE

The term 'neurotoxic' is used to describe a substance, condition or state that damages the nervous system and/or brain, usually by killing neurons. Arsenic is a semi-metalloid and exposure to As is a worldwide health problem causing various disorders and diseases in millions of people around the world. Arsenic causes various diseases such as numerous organ cancers and also patients show severe effects on their nervous system. However, the mechanisms of As

neurotoxicity remain somewhat obscure while the instance of As exposure remains a prevalent human health concern.[114]

Diosmin is a semisynthetic flavone derivative of hesperidin occur naturally in citrus fruits. The drug is widely used in treatment of varicose veins and venous ulcers, lymphatic insuffi ciency and hemorrhoids. In these conditions, Diosmin exerts a venotonic action, decreasing venous reflux, and thereby alleviating edema and providing effective venous drainage.[115]

Moreover, the drug has been shown to provide better outcomes for patients with impaired cardiac function before undergoing cardiac operations that require cardiopulmonary bypass. These effects of Diosmin can be ascribed to the antiinflammatory, microcirculatory, and antioxidant effects of its flavonoid substances. In this context, Diosmin has been shown to decrease the levels of granulocyte and macrophage infi ltration into the infl amed tissues as well as leucocyte adhesion to the vascular endothelium.

The decrease in release of oxygen free radicals, cytokines, and proteolytic matrix metalloproteinases from activated infl ammatory and endothelial cells, results in lower levels of inflammation, decreased microvascular permeability and decreased leukocyte-dependent endothelial damage. Diosmin decreases vascular permeability more than any of its single constituents, suggesting that the flavonoids present in its formulation have a synergistic action.

The drug possesses an antioxidant effect, significantly decreasing the level of hydroxyl free radicals, increasing free SH-group concentration, and natural scavenger capacity.[115]

The effects of arsenic on nervous system have received considerably less attention. In this study we planned to investigate the effects of Arsenic trioxide on the oxidative stress, contents of lipids, proteins, antioxidant defence systems in various regions of the rat brain to seek contribution of arsenic, if any, in peroxidative damage and other neurochemical perturbations. Furthermore, to unravel the effects of recovery on arsenic induced neurotoxicity in various regions of the rat brain.

The present study attempts to screen the arsenic poisoning particularly the role of oxidative stress in the toxic manifestation, an attempt for the treatment and a possible beneficial role of antioxidants supplementation to achieve the optimum effects in wistar rats.

The present work, evaluation of Diosmin against Arsenic induced neurotoxicity was planned with the following objectives:

- To determine the effect of Diosmin on plasma concentrations of total proteins, albumin, glucose, urea, creatinine and bilurubin
- To evaluate Diosmin on the plasma levels of total lipids, cholesterol, triglycerides, HDL-C, LDL-C and VLDL-C
- To study the effect of Diosmin on concentrations of brain and serum Acetylcholinesterase levels
- To determine the effect of Diosmin on Biogenic amines of Corpus striatum, Frontal cortex and Hippocampus in Arsenic trioxide induced neurotoxicity in rats
    - ✓ Dopamine
    - ✓ Epinephrine
    - ✓ Norepinephrine
    - ✓ Serotonin
    - ✓ 3,4-Dihydroxy phenylacetic acid
    - ✓ Homovanilin acid
- To evaluate the effect of Diosmin on Brain tissue Antioxidants in Arsenic trioxide induced neurotoxicity in rats
    - ✓ Lipid peroxidation
    - ✓ Superoxide dismutase
    - ✓ Catalase
    - ✓ Reduced Glutathione
- Histopathological evaluation of brain tissues of rats to evident the neuroprotective nature of Diosmin

4. REVIEW OF LITERATURE

(Yusuf T et al., 2013) Liver ischemia reperfusion injury (IRI) is an important pathologic process leading to bodily systemic effects and liver injury. It has been found that preoperative and intraoperative diosmin treatment decreases cellular damage and protects cells from toxic effects in liver IRI. Diosmin may be used as a protective agent against IRI in elective and emergent liver surgical operations.[116]

(Thiruvengadan Devaki et al., 2013) Hepatic steatosis is obesity related metabolic disorder characterized by aggregation of fat droplets and its regulation is currently being warranted. Disomin was reported to exert antioxidant, anti-mutagenic, anti-hypertensive actions. In this study , we have analyzed the role of diosmin in regulating triglyceride accumulation during high-carbohydrate diet (HCD)-induced hepatic steatosis and acknowledged its role.[117]

(Ivan V. Batchvarov et al., 2010) Chronic venous insufficiency (CVI) is increasingly affecting more and more patients, usually in their prime of life. Patients in all phases of CVI demonstrated good tolerance to the Diosmin, which was found to have the best effect in reducing the edema in patients with initial symptoms. It also had a very good effect on pain intensity in patients feeling heaviness and continuous pains in the lower legs in walking.[118]

(Sergeeva E.O et al., 2011) Hesperidin, diosmin and detraleks demonstrate significant hepatoprotective effect in health-care use in the effective dose against the acute toxic liver injury CCI4 , more effective than the reference drug effects such CARS ekivalentnoy dose. The combination of diosmin and hesperidin 9:1, as in the preparation "Detralex" leads to increased hepatoprotective effect. It can be assumed that the vascular effects, which have hesperidin,diosmin and detraleks, such as, improved microcirculation and metabolism of the vascular wall, are also of great importance for the manifestation of their hepatoprotective activity.[119]

(Maksimovic ZV et al.,) Chronic venous insufficiency (HVI) is manifested by the progressive signs of venous stasis. This disorder is treated by: compressive bandaging, medicaments, sclerotherapy, surgery, etc. in a prospective study administration of semisynthetic diosmin during 30 days results in significant improvement of clinical signs, quality of life and

CEAP stage of HVI.[120]

(Takuji Tanaka et al., 1997) The modifying effects of flavonoids diosmin and hesperidin during the initiation and post-initiation phases of oesophageal carcinogenesis initiated withN-methyl-N-amylnitrosamine (MNAN) were investigated in male Wistar rats. Findings from the study suggest that Diosmin and hesperidin supplementation, individually or in combination, was effective in inhibiting the development of oesophageal cancer induced by MNAN when given during the initiation phase, and such inhibition might be related to suppression of increased cell proliferation caused by MNAN in the oesophageal mucosa.[121]

(Leelavinothan Pari et al., 2010) In the investigation of the effect of diosmin on hepatic key enzymes of carbohydrate metabolism in streptozotocin-nicotinamide-induced diabetic rats, Diosmin was administered to streptozotocin-induced (45 mg/kg b.w) diabetic rats at different doses (25, 50, 100 mg/kg b.w) for 45 days. The activities of the hepatic key enzymes such as hexokinase and glucose-6-phosphate dehydrogenase were significantly increased whereas, glucose-6-phosphatase and fructose-1,6-bisphosphatase were significantly decreased. Furthermore, protection against body weight loss of diabetic animals was also observed. These results showed that diosmin has potential antihyperglycemic activity in streptozotocin-nicotinamide-induced diabetic rats.[122]

(Jean-Pierre Savineau et al., 1994) The effect of diosmin, a flavone derivative, on the Ca2+ sensitivity of the venous contractile apparatus was investigated in chemically (P-escin) skinned strips from the rat isolated femoral vein. Diosmin (0.5-1I0 gM) shifted the concentration-response curve to Ca2" (0.05- 5 gM) to the left. The maximal effect was observed in the presence of 1 J.M diosmin which increased the contractile response evoked by 0.151iM Ca2` from 26.3% to 78.9% of the maximal Ca2+-induced response. This work demonstrates that the venotonic action of diosmin involves an increase in the Ca2+ sensitivity of the contractile apparatus. Such a mechanism of action could represent a new and important means of therapeutic control of vasomotor activity.[123]

(Nuria Alvarez et al., 2008) Melanoma is the most important skin cancer in terms of mortality, besides developing metastasis in about a third of the patients. Interferon-alpha (IFN-a), a highly toxic cytokine, tends to be one of the most important treatments for melanoma. Much effort is being directed at obtaining less-toxic antitumoral compounds, particularly natural

compounds such as flavonoids. In the study of combined treatment of metastatic lung melanoma with IFN-a and diosmin in a murine model, IFN-a showed a dose dependent anti-invasive and anti proliferative activity in our study, while diosmin showed an anti-invasive activity similar to the lower dose of IFN-a used. However, the most relevant result was the synergistic antiproliferative effect shown by the combination of the flavonoid and the lowest dose of IFN-a, which was similar to that produced by the highest dose of the cytokine alone.[124]

(Leelavinothan Pari et al., 2012) Oxidative stress has been suggested as a contributory factor in development and complication of diabetes. In the study of evaluation of effect of diosmin (DS) in oxidative stress in streptozotocinnicotinamide (STZ-NA)-induced diabetic rats by measuring the lipid peroxidation (LPO) as well as the ameliorative properties, oral treatment with DS (100 mg/kg/day) for a period of 45 days showed significant ameliorative effects on all the biochemical parameters studied. Biochemical findings were supported by histological studies. These results indicated that DS has potential ameliorative effects in addition to its antidiabetic effect in type 2 diabetic rats.[125]

(Xingwei Wu et al., 2013) In the evaluation of retinal microvascular protective effect of diosmin in a model of ischemia/reperfusion injury, maintenance of tight junctions integrity and reduced permeability of capillaries as well as improvements in retinal edema were observed with diosmin treatment, which may contribute to preservation of retinal function. This protective effect of diosmin may be at least partly attributed to its ability to regulate the VEGF/PEDF ratio.[126]

(Hye Hyun Yoo et al., 2007) Diosmin is one of the main components in citrus fruits, and the intake of food supplements containing this compound may potentially increase the absorption of drugs able to act as P-gp substrates.[127]

(Anne M. Melin et al., 1996) In the study of comparision of protective effect of a flavonoid, the 3',5,7-trihydroxy- 4'-methoxyflavone 7-rutinoside or diosmin, on liver microsomal lipid peroxidation induced in rats by ei- ther carbon tetrachloride or carrageenan, results suggest that the effect of diosmin differs with the choice of chemical product used; it seems a better antioxidant against products inducing inflammation.[128]

(Ali Noorafshan et al., 2013) In the investigation of evaluation of the effect of Diosmin on renal tissue protection in rats with ethylene glycol-induced nephrolithiasis, Diosmin reduces

CaOx deposition and the degeneration of glomeruli and tubules.[129]

(Martínez1 C et al., 2005) In the study of effect of different phenolic compounds and red wine on pulmonary metastatic melanoma, Diosmin revealed the greatest reduction in pulmonary metastases, both at the macroscopic and microscopic levels.[130]

(Alptekin Yasım et al., 2010) In the investigation of the effect of diosmin-hesperidin combination treatment on serum lipid profile and oxidativeantioxidative system in high-cholesterol diet-fed rats, positive changes occurred in oxidative-antioxidative balance and administration of diosmin-hesperidin significantly increased the levels of GPX and SOD.[131]

5. MATERIALS AND METHODS

Experimental Animals

Male Swiss Albino Wistar rats, weighing 150-180 g were obtained from central animal house of Swamy Vivekanandha College of Pharmacy, Elayampalayam, Tiruchengode, Namakkal Dt, Tamilnadu. The animals were acclimatized for 2 weeks in the laboratory conditions before experiment. Throughout the acclimatization and experimental period, the animals were housed in autoclavable polypropylene cages (six rats per cage) in standard laboratory conditions (humidity 50–60%, lighting conditions (12-h light/12-h dark cycle) and temperature 21 ± 2^{0}C). Rats were provided with standard pellet diet and water *ad libitum* freely throughout the study. Study protocol was approved by Institutional Animal Ethical Committee (IAEC), Swamy Vivekanandha College of Pharmacy (proposal No.SVCP/IAEC/M.Pharm/03/2013) and conducted in accordance with guidelines set by the CPCSEA (Committee for the Purpose of Control and Supervision of Experiment on Animals).

DRUGS

- Diosmin (Sigma-Aldrich Chemicals Pvt. Ltd., Bangalore, India)
- Arsenic Trioxide (Mumbai Chemicals Incha Pvt. Ltd., Mumbai, India)

Experimental Procedure

Administration schedule of Arsenic Trioxide and Diosmin

Total of 24 animals obtained were divided into four groups, where each group under-went different treatment protocol. All animals had free access to regular rat chow and drinking water *ad libitum* for 30 days.[132]

In each major group the treatment schedule for subgroups follows as below

Both ATO and Diosmin were administered individually using water as vehicle (Vol 0.5 ml p.o.)

Group 1: Normal control (N = 6, receives vehicle alone)

Group 2: Receives Arsenic trioxide 5 mg/kg/day p.o. (N = 6)

Group 3: Receives Arsenic trioxide 5 mg/kg p.o. + Diosmin 50 mg/kg p.o. (N = 6)

Group 4: Receives Arsenic trioxide 5 mg/kg p.o. + Diosmin 100 mg/kg p.o. (N = 6)

Collection of blood samples

Rats of each group were euthanized at the end of treatment period. Blood samples were collected from retro orbital venous plexus of rats and placed immediately on ice. Heparin was used as an anticoagulant and plasma samples were obtained by centrifugation at 860g for 20 min and stored at -60 ^{0}C till measurements. Brain was immediately removed; weighed and washed using chilled saline solution. Tissues were minced and homogenized (10%, w/v), separately, in ice-cold 1.15% KCl–0.01 M sodium, potassium phosphate buffer (pH 7.4) in a Potter–Elvehjem type homogenizer. The homogenate was centrifuged at 10,000g for 20 min at 4 ^{0}C, and the resultant supernatant was used for enzyme assays. Stored plasma samples were subjected for biochemical analysis.

BIOCHEMICAL PARAMETERS

Estimation of Plasma biochemistry Lipids and Lipoproteins

1) Estimation of creatinine in plasma

In this study the modified Jaffe's method was used to estimate the creatinine level in plasma.[133]

Principle

Creatinine reacts with picric acid to produce a collared compound, creatinine alkaline picrate. The change in absorbance is proportional to the creatinine concentration.

Procedure

Pipette into tubes marked	**Standard**	**Sample**
Working reagent	1000µL	1000µL
Standard	100µL	-
Sample	-	100µL
Mix and read the optical density (T_1) 60 seconds after the sample or standard addition exactly 60 seconds after the first reading take second reading (T_2)		

2) Estimation of urea in plasma

In this study enzymatic method is used to estimate the urea level in urine and plasma.[134]

Principle

Urease splits urea into ammonia and carbon dioxide. Ammonia released in this reaction reacts with hypochlorite and phenolic chromogen to produce green colour. The absorbance of this green colour at 578 nm (570-620) is directly proportional to the concentration of urea in specimen.

Procedure

Pipette into tubes marked	**Urine/plasma**	**Standard**	**Blank**
Working reagent	0.01 ml	0.01 ml	-
Enzyme solution	1.0 ml	1.0 ml	1.0 ml
Chromogen solution	1.0 ml	1.0 ml	1.0 ml
Mix and incubate for three minutes at 37°C for 5 minutes. After completion of incubation measure the absorbance of assay mixture against blank at 578 nm.			

3) Total protein content in serum [135]

Total protein in serum was measured according to Lowery method as modified by Pomory.

Principle

The principle behind the Lowery method lies in the reactivity of the peptide nitrogen(s) with the copper (II) ions under alkaline conditions which gives a deep blue colour. In addition, subsequent reduction of the folin- ciocalteu phosphomolybdic phosphotungustic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids Thus, producing intense blue-green colour which was measured calorimetrically at 660 nm.

Method

- 1 ml was diluted to 100 ml with 0.1 N NaOH.
- 0.5 ml of plasma, standard protein and 0.1 N NaOH each were pipette out into test tubes labelled 'Test', 'Std' and 'Blank' respectively.
- 5 ml copper-tartarate-carbonate solution was added to each tube, agitated for a few seconds and allowed to stand for 10 min.
- 0.5 ml of 1 N Folin-ciocalteu reagent was then added to all tubes and mixed vigorously allowed standing for 2 h.
- Optical density of intense blue colour produced was measured calorimetrically at 560 nm with UV-Visible spectrophotometer

4) Estimation of Total cholesterol

Method: Chod-Pod/ Phosphotungstate Method [136]

Principle:

Cholesterol + H_2O $\xrightarrow{CHE}$ Cholesterol + Free fatty acid

Cholesterol + O_2 $\xrightarrow{CHOD}$ Cholest - 4ene 3-one + H_2O_2

H_2O_2 + Phenol + 4-amino antipyrine $\xrightarrow{POD}$ $4H_2O$+ Red Quinoneimine complex

CHE – cholesterol esterase

CHOD – cholesterol oxidase

Reagents composition

1.Cholesterol reagent

1	Cholesterol esterase (pancreatic)	>200 IU/L
2	Cholesterol oxidase(microbial)	>150 IU/L
3	Peroxidase (horseradish)	>2000 IU/L
4	Sodium phenolate	20 m mol/L
5	4-amino antipyrine	0.5 m mol/L
6	Phosphate buffer	68 m mol/L
7	Lipid clearing agent	------

2. Cholesterol standard - 200 mg/dl (5.14 mmol/L)

Assay and procedure

Fresh clear and unhaemolysed serum was used for the estimation.

Assay parameters

Mode	Kinetic
Wavelength(1nm)	505nm
Wavelength (2nm)	670nm
Sample volume	10/20µl
Reagent volume	500/1000µl
Incubation time	10
Incubation temperature	37°C
Normal low	140
Normal High	250
Linearity low	0
Linearity high	600
Conc. of standard	200
Blank	Reagent
Absorbance limit	0.4
Units	mg/dl

Assay Procedure

Pipette into tubes marked	**Blank**	**Standard**	**Test**
Serum/plasma	-	-	20µl
Cholesterol standard	-	20 µl	-
cholestrol reagent	1000 µl	1000 µl	1000 µl

Mixed well and incubated for 5 min at 37°C or 10 min at room temperature then the samples were analyzed in semi auto analyzer.

5) Estimation of TG (Triglycerides) [136]

Method: (GPO-trinder method)

Principle:

Triglycerides + H_2O $\xrightarrow{\text{Lipases}}$ Glycerol + fatty acids

Glycerol +ATP $\xrightarrow{\text{GPO}}$ Glycerol – 3-phoshate (G-3-P) + ADP

G-3-P + O_2 $\xrightarrow{\text{GPO}}$ Dihydroxy acetone phosphate+H_2O_2

H_2O_2 + 4-amino antipyrine + 3,5 – DHBS $\xrightarrow{\text{Peroxidase}}$ Quinoneiminedye + H_2O

GPO – Glycerol phosphate oxidase

DAP – Dihydroxyacetone phosphate

DHBS – 3,5-dichloro-2-hydroxybenzene sulfonate

Reagents composition

1.Triglycerides des reagent

s.no	**Active ingredient**	**Concentration**
1	ATP	2.5 m mol/L
2	Mg^{2+}	2.5 m mol/L
3	4-amino antipyrine	0.8 m mol/L
4	3-5 DHBS	1 m mol/L
5	Peroxidase	>2000 IU/L
6	Glycerol kinase	>550 IU/L
7	GPO	>8000 IU/L
8	Lipoprotien lipase	>3500 IU/L
9	Buffer (p^H 7.0± 0.1 at 20^0C	53 m mol/L

Also contains non-reactive filers, stabilisers and surfactants.

Triglycerides standard - 200 mg/dl (2.3 m mol/L)

Assay parameters:

Mode	Kinetic
Wavelength(1nm)	505nm
Wavelength (2nm)	670nm
Sample volume	5/10
Reagent volume	500/1000
Incubation time	10min
Incubation temperature	37
Normal low	25 mg/dl
Normal High	160 mg/dl
Linearity low	0 mg/dl
Linearity high	900mg/dl
Conc. of standard	200mg/dl
Blank	Reagent
Absorbance limit	0.4
Units	mg/dl

Procedure

Pipette into 3 test tubes labeled blank (B), standard(S), and total cholesterol (T_c) as shown below.

Pipette into tube marked	**Blank**	**Standard**	**Test**
Working Reagent	1000 µl	1000 µl	1000 µl
Standard	-	10 µl	-
Specimen	-	-	10µl

Mixed well and incubated for 10 minutes at $37^{0\ c}$ or 15 min at room temperature then the samples were analyzed in semi auto analyzer.

6) Estimation of HDL (High density lipoproteins) [136]

Principle

Serum $\xrightarrow{\text{phosphotunstate}}$ HDL + LDL +VLDL + Chylomicrons

On addition of precipitating reagent to the serum, followed by centrifugation, HDL fraction remains in the supernatant while the lipoproteins precipitate out.

Reagents composition

1. Precipitating reagent

1	Phoshotunstic acid	2.4 m mol/L
2	Magnesium chloride	40 m mol/L

2. HDL cholesterol standard – 25 mg/dl.

Assay and procedure

Fresh clear and unhaemolysed serum was used for the estimation

Assay parameters

For estimation with cholesterol reagent

Mode	End Point
Wavelength(1nm)	505nm
Wavelength (2nm)	670nm
Sample volume	25/50µl
Reagent volume	500/1000µl
Incubation time	10 min
Incubation temperature	37^0C
Normal low	30mg/dl
Normal High	80mg/dl
Linearity low	0mg/dl
Linearity high	125mg/dl
Conc. of standard	25mg/dl
Blank	Reagent
Absorbance limit	0.3
Units	mg/dl

Procedure

Step1: Pipette into the centrifuge tube.

Serum / plasma	0.2 ml
Precipitating reagent (3)	0.3 ml

Mixed well and allowed standing at room temperature for 5 min. Centrifuged at 3000 rpm for 10 min to get a clear supernatant. If supernatant is not clear (high TGL level) dilute the sample 1:1 normal saline and multiply the result with 2.

Step 2: Pipette into 3 test tubes labeled blank (B), standard (S), HDL cholesterol (T_H) as shown below.

Pipette into tube marked	**Blank**	**Standard**	**Test**
Working Reagent	1000 µl	1000 µl	1000 µl
Standard	-	50 µl	-
Supernatant	-	-	50µl

Mixed well and incubated for 5 min at 37°C or 10 min at room temperature then the samples were analyzed in semi auto analyzer.

7) **LDL cholesterol** = Total cholesterol–(HDL cholesterol + TG/5)

8) VLDL cholesterol= TG/5

***In vivo* antioxidant parameters**

Preparation of homogenate

Procedure

Brains were excised and chopped with surgical scalp into fine slices and were chilled in the cold 0.25 M sucrose, quickly blotted with filter paper. The tissue was minced and homogenized in ice cold 10 mM tris HCl buffer (to pH 7.4) at a concentration of 10% (w/v) with 25 stokes of tight teflon pestle of glass homogenizer at a speed of 2500 rpm. The prolonged

homogenization under hypotonic condition was designed to disrupt as far as possible the ventricular structure of cells so as to release soluble protein and leave only membrane and non-vascular matter in a sedimentable form. It was then centrifuged at 5000 rpm at 20^{o} C temperature and clear supernatant was separated and used to estimate reduced glutathione (GSH), catalase (CAT) and lipidperoxidation (LPO).

1) Estimation of Lipid peroxidation [137]

Lipid peroxidation was determined by the method of Slater and Sawsyer *et al.,* 1971. The large number of polyunsaturated fatty acids (PUFA's) makes cell membranes particularly vulnerable to lipid peroxidation. The oxidation of PUFA causes them to be more hydrophilic, thereby altering the structure of membrane with resultant changes in fluidity and permeability. Lipid peroxidation can inhibit the function of membrane bound receptors and enzymes. The thiobarbituric acid reacting substance (TBARS) assay is used as an indicator of lipid peroxidation and levels of free radicals (Sun and Chen, 1998).

Reagents

1. Thiobarbituric acid: 0.67% w/v in 1M tris hydrochloride pH 7, 0.67 g of thiobarbituric acid was dissolved in 100 ml of distilled water.
2. Trichloroacetic acid (20% w/v): 20 g of TCA was dissolved in 100 ml of distilled water.
3. Standard malondialdehyde (0-25 n.mol)

A stock solution containing 50 mm/ml of 1, 1, 3, 3 - tetra ethoxy propane in tris hydrochloride buffer in pH -7, 10 ml of stock solution was diluted to 100 ml to get a working standard 50 nm malondialdehyde/ml. This was used for preparation of calibration curves.

Procedure

2 ml of sample was mixed with 2 ml of 20% TCA and kept in ice for 15 min. The precipitate was separated by centrifugation and 2 ml of samples of clear supernatant solution were mixed with 2 ml aq. 0.67% TBA solution. This mixture was heated on a boiling water bath for 10 min. It was cooled in ice for 5 min and absorbance was read at 535 nm. The values were expressed as nm of MDA formed/mg of protein values are normalized to protein content of tissues.

$$X = \frac{(Y + 0.002)}{0.0026086}$$

Y – Absorbance differences of final (after 3 min) and initial reading of test sample.

2) Superoxide dismutase [138]

The SOD activity in supernatant was measured by the method of Misra and Fridovich. Superoxide dismutase is an important endogenous antioxidant and prevents production of free radicals. It is derived from various sources at different stages of reperfusion (Chaudhary G *et al*., 2003).

Reagents

1. Carbonate buffer (100mM, pH 10.2)
2. Epinephrine (3mM) is prepared by reconstituting one vial with 1.0 mL of distilled or deionized water. Mix gently until completely dissolved.

Procedure

The supernatant (500 µl) was added to 0.800ml of carbonate buffer (100mM, pH 10.2) and 100 µl of epinephrine (3mM). The change in absorbance of each sample was then recorded at 480 nm in spectrophotometer for 2 min at an interval of 15 sec. Parallel blank and standard were run for determination SOD activity.

One unit of SOD is defined as the amount of enzyme required to produce 50% inhibition of epinephrine auto oxidation.

Reagents	**Uninhibited(Standard)**	**Inhibited(Sample)**	**Blank**
Carbonate buffer	0.900 ml	0.800 ml	1.0 ml
Supernatant	__	0.1 ml	__
Epineprine	0.1 ml	0.1 ml	__

The reaction mixtures are diluted 1/10 just before taking the readings in spectrophotometer.

Calculation

$$\% \text{ Inhibition} = \frac{A_{480nm}/\text{min Uninhibited} - A_{480nm}/\text{min Inhibited}}{A_{480nm}/\text{min Uninhibited} - A_{480nm}/\text{min Blank}} \times 100$$

$$\text{Units/ml enzyme} = \frac{\% \text{ Inhibition} \times V_t}{(50\%) \times V_s}$$

$$\text{Units/mg protein} = \frac{\text{Units/ml enzyme}}{\text{mg protein/ml enzyme}}$$

Where A = Absorbance, V_t = total volume, V_s = sample volume.

3) Reduced glutathione (GSH) [139]

Reduced glutathione was determined by the method of Moran *et al.,*1979. Normally, the brain maintains a high ratio of GSH for antioxidant defense. Depletion of total glutathione and a decreased GSH are markers for oxidative stress in ischemic brain (Park et al., 2000).

Reagents

1. TCA (10% w/v) solution: Accurately weighed 10 g of TCA was dissolved in 100 ml of distilled water.
2. Phosphate buffer (0.2 M, pH 8)
3. DTNB reagent (0.6 M): 60 mg of 5,5-dithio bis (2-nitro benzoic acid) was dissolved in 100 ml of 0.2 M sodium phosphate (pH 8).
4. Standard glutathione: Prepared by dissolving 10 mg of reduced glutathione in 100 ml of distilled water.

Procedure

To 1 ml of sample, 1 ml of 10% TCA was added. The precipitated fraction was centrifuged and to 0.5 ml supernatant, 2 ml DTNB was added. The final volume was made up to 3 ml with phosphate buffer. The colour developed was read at 412 nm. The amount of glutathione was expressed as µg of GSH/mg protein reduced glutathione was used as standard (100 µg/ml).

$$X = \frac{(Y - 0.0046)}{0.0034}$$

Y – Absorbance of test sample.

4) Catalase (CAT)

Catalase was estimated by the method of Hugo E. Aebi method: hydrogen peroxide: hydrogen-peroxidoreductase. Catalase decomposes hydrogen peroxidase and converts it to water and diatomic oxygen, whereas superoxide dismutase generates H_2O_2 from free radicals. An increase in production of superoxide dismutase without a subsequent elevation of catalase leads to the accumulation of hydrogen peroxidase, which is converted to hydroxyl radicals that produced deleterious effect on brain (Pigeolet, 1998).

Principle

In UV range H_2O_2 can be followed directly by the decrease in absorbence (O.D 240) per unit time is measure of catalase activity.

$H_2O_2 \longrightarrow H_2 + O_2$

$RDOH \longrightarrow H_2O + ROH + A$

Decomposition of H_2O_2 = Decrease in absorbance at 240 nm

Reagents

1. Phosphate buffer (50 mM, pH 7.0)

A. Dissolve 6.81 g KH_2PO_4 in distilled water and make up to 1000 ml.

B. Dissolve 8.9 g NaH_2PO_4. $2H_2O$ in distilled water and make up to 1000 ml.

Mix the solution A and B in proportion 1:15 (v/v)

2. Hydrogen peroxide (30 mM/I): Dilute 0.34 ml of 30% Hydrogen peroxide with phosphate buffer up to 100 ml.

Procedure

Dilute homogenate 20 times with Phosphate buffer pH 7.0

Blank	**Test**
4 ml of homogenate diluted with 2 ml of phosphate buffer P^H 7, and take absorbance at 254 nm for 3 min. with 30 sec. interval	2 ml of homogenate diluted with 1 ml of H_2O_2 (8.5 micro lit. in 2.5 ml phosphate buffer (50mM/l. pH 7.0) and take the absorbance at 254 nm for 3 min. with 30 sec. interval. (Add H_2O_2 just before taking O.D)

Calculation

Log (A / B) × 2297.3

Where, A: Initial absorbance

B: final absorbance (after 30 second)

Units = μ moles of H_2O_2 consumed/min/mg

Histopathological studies

For Histopathological studies, brain tissue was obtained from the excised brain and was immediately fixed in 10% buffered neutral formalin solution. The fixed tissues were embedded in paraffin and serial sections were cut. Each section was stained with hematoxylin and eosin (H & E stain). The sections were examined under light microscope and photomicrographs were taken.

Statistical analysis

In the present study, all the data was expressed as mean ± S.E.M. Statistical significance between more than two groups was tested using one way ANOVA followed by the Tukey test using computer based fitting program (Prism graph pad). Statistical significance was set accordingly.

6. RESULTS

The neuroprotective activity was evaluated in Swiss Albino Wistar Rats. Where Group 1 kept as Normal control, Group 2 received Arsenic trioxide 5 mg/kg p/o, group 3 received Arsenic trioxide 5 mg/kg + Diosmin 50 mg/kg p/o and group 4 received Arsenic trioxide 5 mg/kg + Diosmin 100 mg/kg p/o. at the end of study several parameters like Serum Biochemistry, AchE levels, Brain tissue antioxidant levels and Histopathology were evaluated.

Table No. 1 Effect of Diosmin on animal body weights in Arsenic trioxide induced neurotoxicity in rats

Table No. 2 Effect of Diosmin on brain weights in Arsenic trioxide induced neurotoxicity in rats

Table No. 3 Effect of Diosmin on plasma biochemistry in Arsenic trioxide induced neurotoxicity in rats

Table No. 4 Effect of Diosmin on plasma lipids and lipoproteins in Arsenic trioxide induced neurotoxicity in rats

Table No. 5 Effect of Diosmin on Brain and Serum Acetylcholinesterase levels in Arsenic trioxide induced neurotoxicity in rats

Table No. 6 Effect of Diosmin on Biogenic amines of Corpus striatum in Arsenic trioxide induced neurotoxicity in rats

Table No. 7 Effect of Diosmin on Biogenic amines of Frontal cortex in Arsenic trioxide induced neurotoxicity in rats

Table No. 8 Effect of Diosmin on Biogenic amines of Hippocampus in Arsenic trioxide induced neurotoxicity in rats

Table No. 9 Effect of Diosmin on Brain tissue Antioxidants in Arsenic trioxide induced neurotoxicity in rats

Graphs showing the statistical analysis of various parameters

Graph No.1 Effect of Diosmin on animal body weights in Arsenic trioxide induced neurotoxicity in rats

Graph No. 2 Effect of Diosmin on brain weights in Arsenic trioxide induced neurotoxicity in rats

Graph No.3 Effect of Diosmin on plasma biochemistry (Total Proteins, Albumin,Glucose, Urea) in Arsenic trioxide induced neurotoxicity in rats

Graph No.4 Effect of Diosmin on plasma biochemistry (Creatinine, Bilirubin) in Arsenic trioxide induced neurotoxicity in rats

Graph No.5Effect of Diosmin on plasma lipids and lipoproteins (Total lipids, Cholesterol, Triglycirides, LDL-C) in Arsenic trioxide induced neurotoxicity in rats

Graph No.6 Effect of Diosmin on plasma lipids and lipoproteins (HDL-C and VLDL-C) in Arsenic trioxide induced neurotoxicity in rats

Graph No.7 Effect of Diosmin on Brain and Serum Acetylcholinesterase levels in Arsenic trioxide induced neurotoxicity in rats

Graph No. 8 Effect of Diosmin on Biogenic amines (Norepinephrine, Epinephrine, Serotonin, Homovanilic acid) of Corpus striatum in Arsenic trioxide induced neurotoxicity in rats

Graph No.9 Effect of Diosmin on Biogenic amines (Dopamine and 3,4- Dihydroxy Phenyl acetic acid) of Corpus striatum in Arsenic trioxide induced neurotoxicity in rats

Graph No.10 Effect of Diosmin on Biogenic amines (Dopamine, 3,4- Dihydroxy Phenyl acetic acid, Norepinephrine and epinephrine) of Frontal cortex in Arsenic trioxide induced neurotoxicity in rats

Graph No.11 Effect of Diosmin on Biogenic amines (Serotonin and Homovanilic acid) of Frontal cortex in Arsenic trioxide induced neurotoxicity in rats

Graph No.12 Effect of Diosmin on Biogenic amines (Dopamine, Serotonin, Norepinephrine, Epinephrine) of Hippocampus in Arsenic trioxide induced neurotoxicity in rats

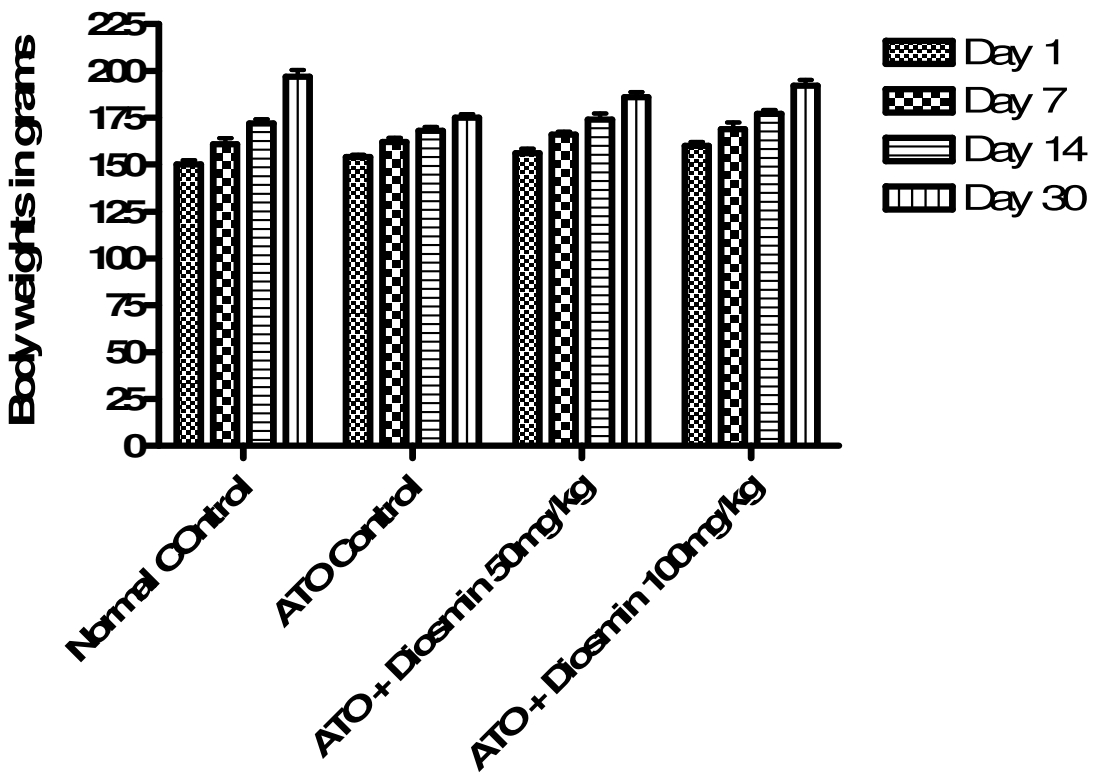
Table No. 1 Effect of Diosmin on animal body weights in Arsenic trioxide induced neurotoxicity in rats

S.No	Treatment	Day 1	Day 7	Day 14	Day 28
1	**Normal control**	150 ± 2.3	161 ± 3.2	172 ± 2.3	197 ± 3.6
2	**Arscenic trioxide (5mg/kg)**	154 ± 1.4	162 ± 2.3	168 ± 2.1*	175 ± 1.9**
3	**Arscenic trioxide (5mg/kg) + Diosmin (50mg/kg)**	156 ± 2.5	166 ± 1.5	174 ± 3.3#	186 ± 2.8#
4	**Arscenic trioxide (5mg/kg) + Diosmin (100mg/kg)**	160 ± 2.0	169 ± 3.5	177 ± 2.1#	192 ± 3.2##

N = 6; values were expressed as Mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 when compared to normal control; #p<0.05, #p<0.01, ###p<0.001 when compared to arsenic trioxide treatment group. Data were analysed by one way ANOVA followed by Tukey's multiple comparision test.

Daily examination of bodyweights in all the treatment groups during the study showed, reduced weight gain in ATO control group when compared to normal control and drug treated groups.

Graph No. 1 Effect of Diosmin on animal body weights in Arsenic trioxide induced neurotoxicity in rats



N = 6; values were expressed as Mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 when compared to normal control; #p<0.05, #p<0.01, ###p<0.001 when compared to arsenic trioxide treatment group. Data were analysed by one way ANOVA followed by Tukey's multiple comparision test.

Animals in ATO control group showed a reduced body weight gain when compared to other groups in the scheduled treatment.

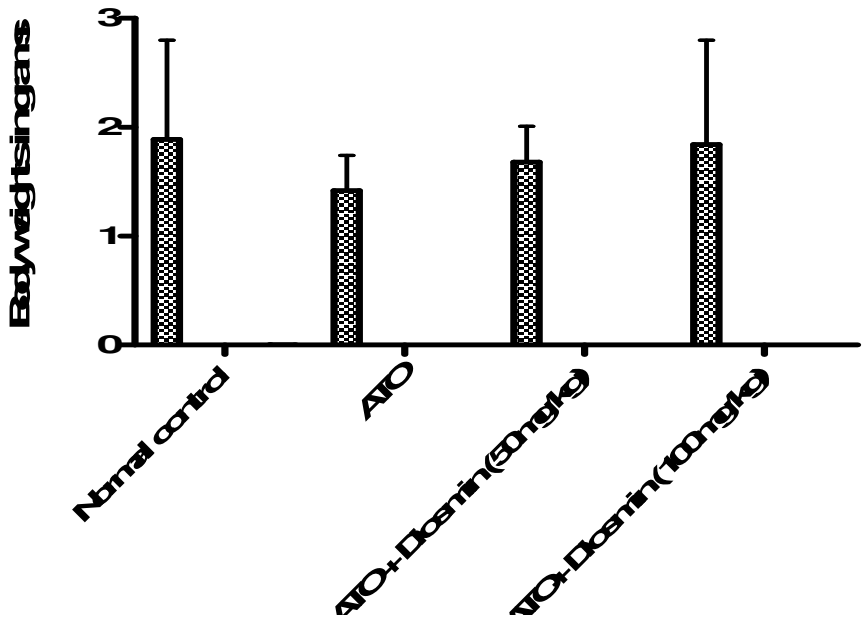
Table No. 2 Effect of Diosmin on brain weights in Arsenic trioxide induced neurotoxicity in rats

S.No	Treatment	Brain weights (gm)
1	Normal controlTotal PRoteins	1.89 ± 0.91
2	Arscenic trioxide (5mg/kg)	1.42 ± 0.32**
3	Arscenic trioxide (5mg/kg) + Diosmin (50mg/kg)	1.68 ± 0.33#
4	Arscenic trioxide (5mg/kg) + Diosmin (100mg/kg)	1.84 ± 0.96##

N = 6; values were expressed as Mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 when compared to normal control; #p<0.05, #p<0.01, ###p<0.001 when compared to arsenic trioxide treatment group. Data were analysed by one way ANOVA followed by Tukey's multiple comparision test.

Measuring the weights of fresh brains just after the isolation at the end of treatment showed that ATO control animals has a reduced weight when compared to other groups.

Graph No. 2 Effect of Diosmin on brain weights in Arsenic trioxide induced neurotoxicity in rats



N = 6; values were expressed as Mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 when compared to normal control; #p<0.05, #p<0.01, ###p<0.001 when compared to arsenic trioxide treatment group. Data were analysed by one way ANOVA followed by Tukey's multiple comparision test.

This graph explains the declined brain weights that were observed at the end of treatment.

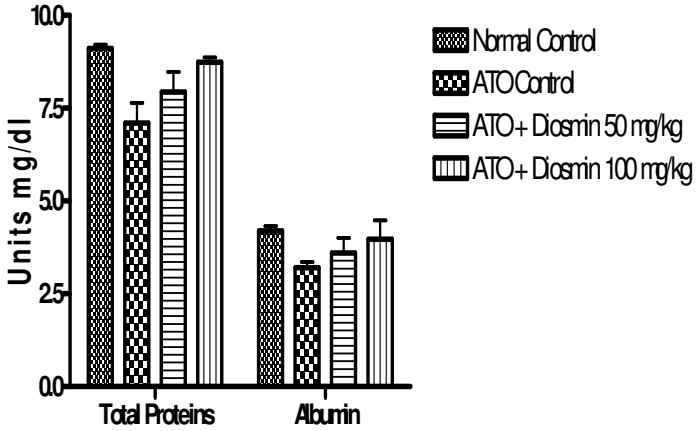
Table No. 3 Effect of Diosmin on plasma biochemistry in Arsenic trioxide induced neurotoxicity in rats

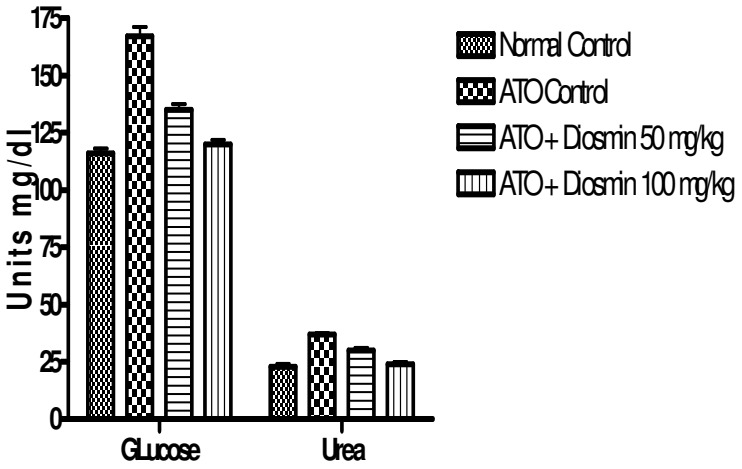
S. No	Treatment	Parameters (mg/dl)					
		Total proteins	**Albumin**	**Glucose**	**Urea**	**Creatinine**	**Bilurubin**
1.	**Normal control**	9.1 ± 0.11	4.2 ± 0.12	116 ± 2.1	22.8 ± 1.4	0.35 ± 0.02	0.64 ± 0.04
2.	**Arscenic trioxide (5mg/kg)**	7.1 ± 0.54**	3.2 ± 0.15***	167 ± 4.1***	37 ± 0.5***	0.71 ± 0.02***	1.24 ± 0.05***
3.	**Arscenic trioxide (5mg/kg) + Diosmin (50mg/kg)**	7.94 ± 0.54*$^{\#}$	3.6 ± 0.40*$^{\#}$	135 ± 2.4**$^{\#\#}$	30 ± 1.2**$^{\#}$	0.58 ± 0.04**$^{\#}$	0.95 ± 0.01***
4.	**Arscenic trioxide (5mg/kg) + Diosmin (100mg/kg)**	8.74 ± 0.12$^{\#\#}$	3.97 ± 0.51$^{\#\#\#}$	120 ± 1.9$^{\#\#\#}$	24 ± 0.9$^{\#\#\#}$	0.40 ± 0.03$^{\#\#\#}$	0.70 ± 0.05$^{\#\#\#}$

N = 6; values were expressed as Mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 when compared to normal control; #p<0.05, #p<0.01, ###p<0.001 when compared to arsenic trioxide treatment group. Data were analysed by one way ANOVA followed by Tukey's multiple comparision test.

Animals treated with Diosmin 100 mg/kg showed a significant effect on plasma biochemistry in animals given with ATO.

Graph No. 3 Effect of Diosmin on plasma biochemistry (Total Proteins, Albumin,Glucose, Urea) in Arsenic trioxide induced neurotoxicity in rats

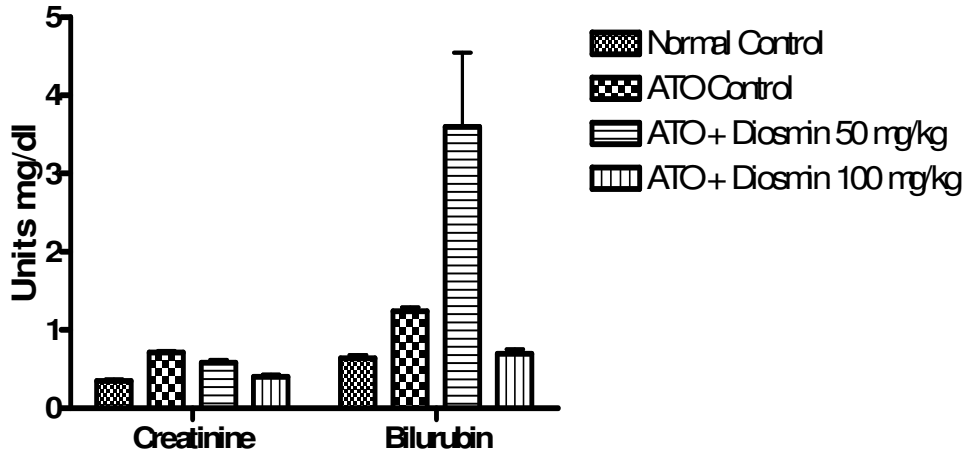




N = 6; values were expressed as Mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 when compared to normal control; #p<0.05, #p<0.01, ###p<0.001 when compared to arsenic trioxide treatment group. Data were analysed by one way ANOVA followed by Tukey's multiple comparision test.

This graph explains the protective nature of Diosmin on plasma proteins, glucose, urea and albumin.

Graph No. 4 Effect of Diosmin on plasma biochemistry (Creatinine, Bilirubin) in Arsenic trioxide induced neurotoxicity in rats



N = 6; values were expressed as Mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 when compared to normal control; #p<0.05, #p<0.01, ###p<0.001 when compared to arsenic trioxide treatment group. Data were analysed by one way ANOVA followed by Tukey's multiple comparision test.

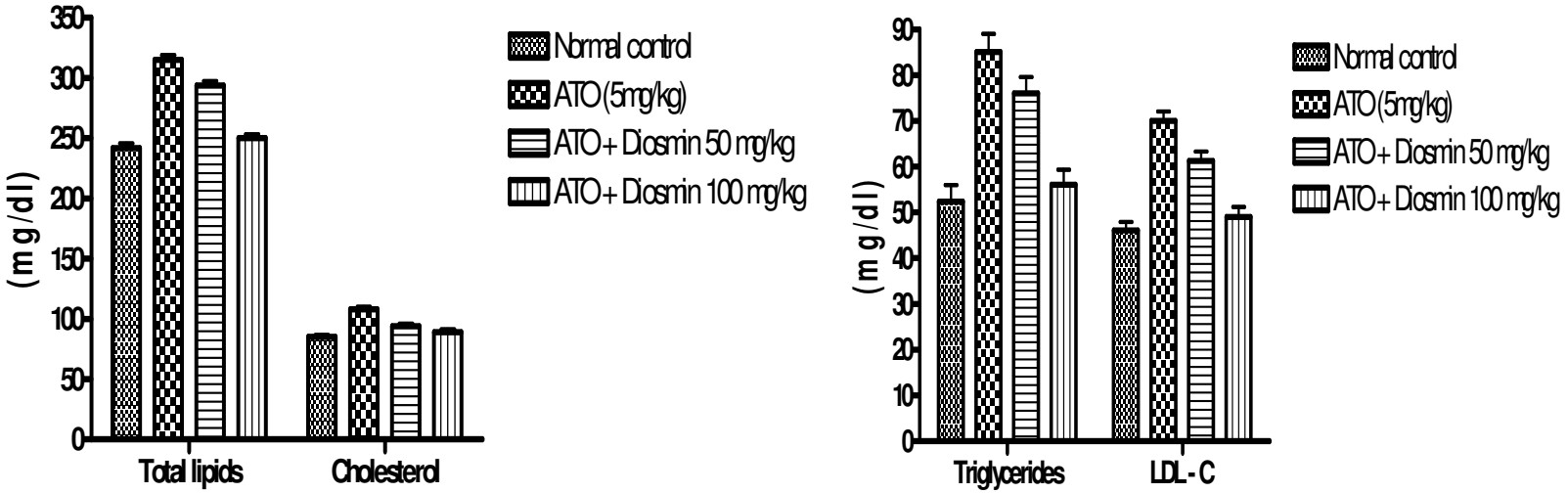
This graph explains the protective nature of Diosmin on plasma proteins, glucose, urea and albumin.

Table No. 4 Effect of Diosmin on plasma lipids and lipoproteins in Arsenic trioxide induced neurotoxicity in rats

S. No	Treatment	Parameters (mg/dl)					
		Total lipids	**Cholesterol**	**Triglycerides**	**HDL - C**	**LDL - C**	**VLDL – C**
1.	**Normal control**	242 ± 3.71	85 ± 1.90	52.31 ± 2.11	26.01 ± 1.31	46.01 ± 1.91	11.23 ± 0.57
2.	**Arscenic trioxide (5mg/kg)**	315 ± 4.01**	108 ± 2.0**	85.00 ± 3.40***	19.01 ± 1.50	70.01 ± 2.40***	36.00 ± 1.02***
3.	**Arscenic trioxide (5mg/kg) + Diosmin (50mg/kg)**	294 ± 3.50*$^{\#}$	94 ± 2.10*$^{\#}$	76.01 ± 3.21*$^{\#}$	21.02 ± 1.32*	61.20 ± 2.01*$^{\#}$	20.05 ± 1.03**$^{\#\#}$
4.	**Arscenic trioxide (5mg/kg) + Diosmin (100mg/kg)**	250.1 ± 3.21$^{\#\#\#}$	89.01 ± 2.20$^{\#\#}$	56.04 ± 2.91$^{\#\#\#}$	25.21 ± 1.42$^{\#\#}$	49.02 ± 2.12$^{\#\#\#}$	12.54 ± 1.01$^{\#\#\#}$

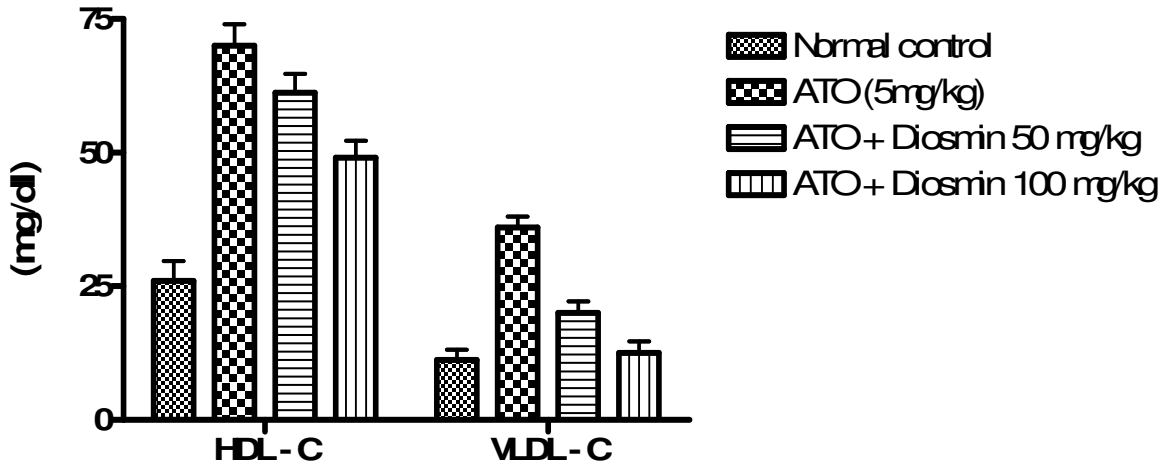
N = 6; values were expressed as Mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 when compared to normal control; #p<0.05, #p<0.01, ###p<0.001 when compared to arsenic trioxide treatment group. Data were analysed by one way ANOVA followed by Tukey's multiple comparision test.

Graph No. 5 Effect of Diosmin on plasma lipids and lipoproteins (Total lipids, Cholesterol, Triglycirides, LDL-C) in Arsenic trioxide induced neurotoxicity in rats



N = 6; values were expressed as Mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 when compared to normal control; #p<0.05, #p<0.01, ###p<0.001 when compared to arsenic trioxide treatment group. Data were analysed by one way ANOVA followed by Tukey's multiple comparision test.

Graph No. 6 Effect of Diosmin on plasma lipids and lipoproteins (HDL-C and VLDL-C) in Arsenic trioxide induced neurotoxicity in rats



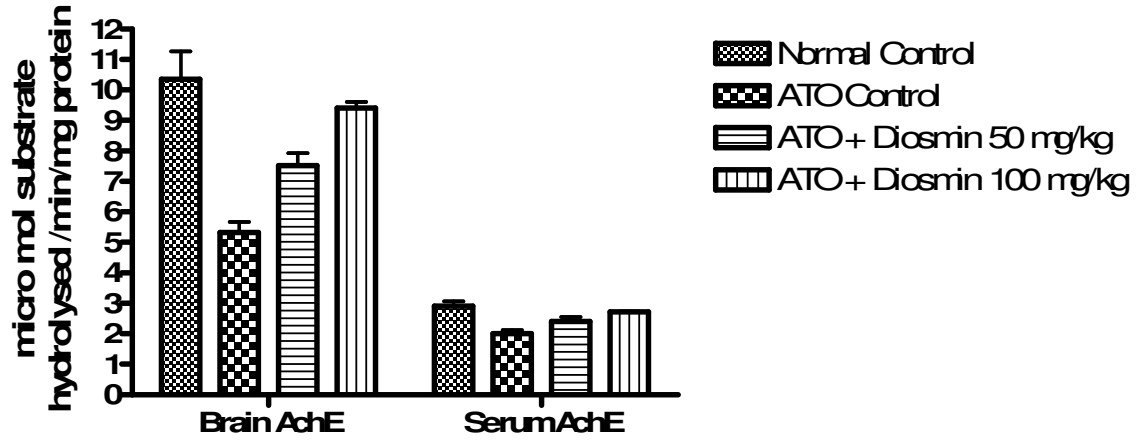
N = 6; values were expressed as Mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 when compared to normal control; #p<0.05, #p<0.01, ###p<0.001 when compared to arsenic trioxide treatment group. Data were analysed by one way ANOVA followed by Tukey's multiple comparision test.

Table No. 5 Effect of Diosmin on Brain and Serum Acetylcholinesterase levels in Arsenic trioxide induced neurotoxicity in rats

S. No	Treatment	Brain AchE (µ mol substrate hydrolysed/ min/mg proteins)	Serum AchE (µ mol substrate hydrolysed/min)
1.	**Normal control**	10.35 ± 0.91	2.91 ± 0.15
2.	**Arscenic trioxide (5mg/kg)**	5.32 ± 0.35***	2.01 ± 0.12*
3.	**Arscenic trioxide (5mg/kg) + Diosmin (50mg/kg)**	7.52 ± 0.41*#	2.40 ± 0.15##
4.	**Arscenic trioxide (5mg/kg) + Diosmin (100mg/kg)**	9.41 ± 0.202###	2.72 ± 0.01###

N = 6; values were expressed as Mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 when compared to normal control; #p<0.05, #p<0.01, ###p<0.001 when compared to arsenic trioxide treatment group. Data were analysed by one way ANOVA followed by Tukey's multiple comparision test.

Graph No.7 Effect of Diosmin on Brain and Serum Acetylcholinesterase levels in Arsenic trioxide induced neurotoxicity in rats



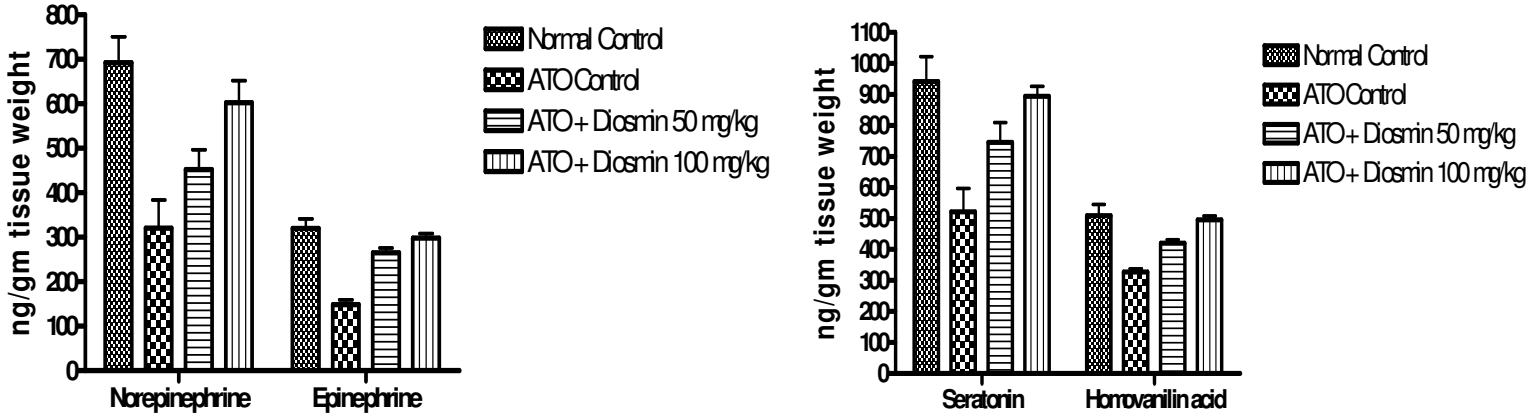
N = 6; values were expressed as Mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 when compared to normal control; #p<0.05, #p<0.01, ###p<0.001 when compared to arsenic trioxide treatment group. Data were analysed by one way ANOVA followed by Tukey's multiple comparision test.

Table No. 6 Effect of Diosmin on Biogenic amines of Corpus striatum in Arsenic trioxide induced neurotoxicity in rats

S. No	Treatment	Biogenic amines (ng/gm tissue weight)					
		Dopamine	**Norepinephrine**	**Epinephrine**	**serotonin**	**3, 4 - dihydroxy phenyl acetic acid**	**Homovanilic acid**
1.	**Normal control**	4395 ± 148	692 ± 59.20	320 ± 21.50	941 ± 81	3120 ± 192	509 ± 35.81
2.	**Arscenic trioxide (5mg/kg)**	3042 ± 152***	321 ± 63.31***	149 ± 10.90***	521 ± 75***	2257 ± 160***	328 ± 10.02***
3.	**Arscenic trioxide (5mg/kg) + Diosmin (50mg/kg)**	3951 ± 160*$^{\#}$	452 ± 45.01$^{\#\#}$	265 ± 11.05$^{\#\#}$	745 ± 64$^{\#\#}$	2850 ± 152*$^{\#\#\#}$	420 ± 11.05*$^{\#\#}$
4.	**Arscenic trioxide (5mg/kg) + Diosmin (100mg/kg)**	4320 ± 150$^{\#\#\#}$	602 ± 50.01$^{\#\#}$	298 ± 10.11$^{\#\#\#}$	894 ± 32$^{\#\#}$	3010 ± 142$^{\#\#\#}$	495 ± 12.11$^{\#\#\#}$

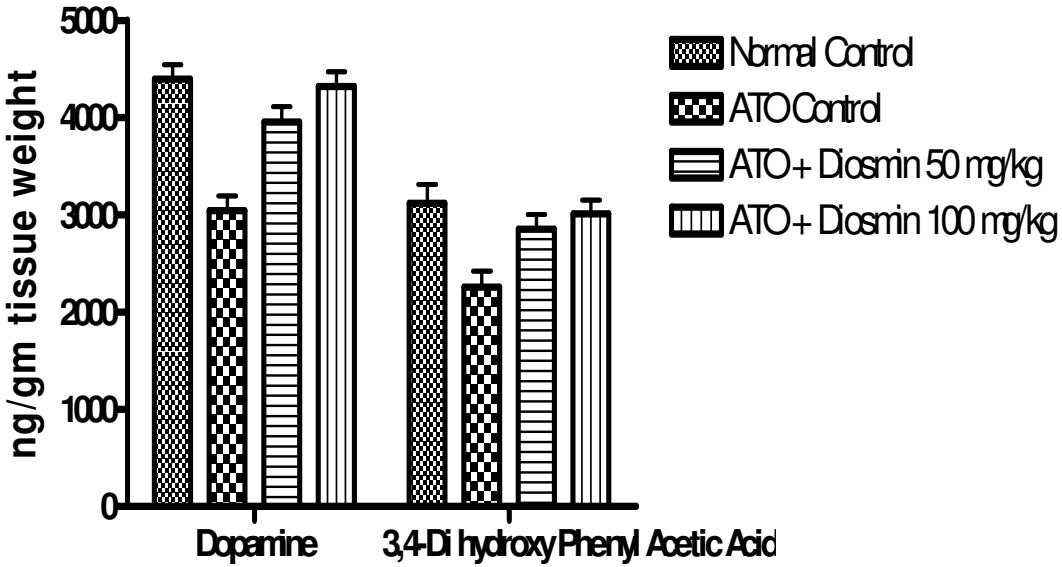
N = 6; values were expressed as Mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 when compared to normal control; #p<0.05, #p<0.01, ###p<0.001 when compared to arsenic trioxide treatment group. Data were analysed by one way ANOVA followed by Tukey's multiple comparision test.

Graph No. 8 Effect of Diosmin on Biogenic amines (Norepinephrine, Epinephrine, Serotonin, Homovanilic acid) of Corpus striatum in Arsenic trioxide induced neurotoxicity in rats



N = 6; values were expressed as Mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 when compared to normal control; #p<0.05, #p<0.01, ###p<0.001 when compared to arsenic trioxide treatment group. Data were analysed by one way ANOVA followed by Tukey's multiple comparision test.

Graph No. 9 Effect of Diosmin on Biogenic amines (Dopamine and 3,4- Dihydroxy Phenyl acetic acid) of Corpus striatum in Arsenic trioxide induced neurotoxicity in rats



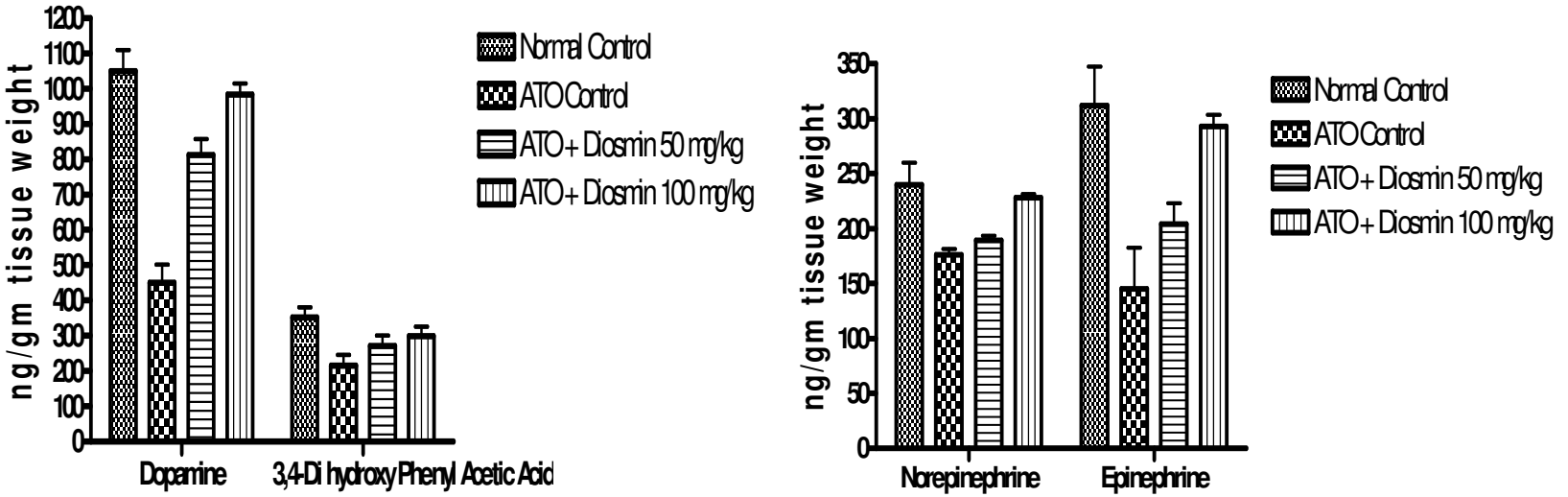
N = 6; values were expressed as Mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 when compared to normal control; #p<0.05, #p<0.01, ###p<0.001 when compared to arsenic trioxide treatment group. Data were analysed by one way ANOVA followed by Tukey's multiple comparision test.

Table No. 7 Effect of Diosmin on Biogenic amines of Frontal cortex in Arsenic trioxide induced neurotoxicity in rats

S. No	Treatment	Biogenic amines (ng/gm tissue weight)					
		Dopamine	**Norepinephrine**	**Epinephrine**	**serotonin**	**3, 4 dihydroxy phenyl acetic acid**	**Homovanilic acid**
1.	**Normal control**	1050 ± 60.12	240 ± 20.01	312 ± 35.12	769 ± 25.42	351 ± 29.12	95 ± 4.24
2.	**Arscenic trioxide (5mg/kg)**	450 ± 51.02***	176 ± 5.42***	145 ± 37.42***	541 ± 26.42***	215 ± 30.10***	56 ± 3.17**
3.	**Arscenic trioxide (5mg/kg) + Diosmin (50mg/kg)**	812 ± 45.12*###	189 ± 4.40*	204 ± 19.11##	623 ± 25.91##	270 ± 30.13##	70 ± 4.02*##
4.	**Arscenic trioxide (5mg/kg) + Diosmin (100mg/kg)**	984 ± 31.05###	228 ± 3.40###	293 ± 10.54###	729 ± 25.54###	298 ± 27.42*##	89 ± 5.16###

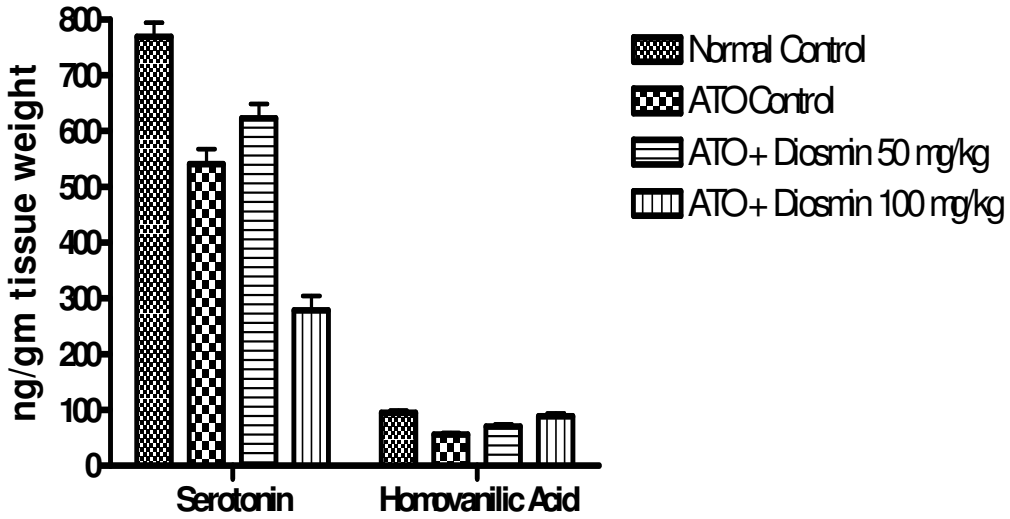
N = 6; values were expressed as Mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 when compared to normal control; #p<0.05, #p<0.01, ###p<0.001 when compared to arsenic trioxide treatment group. Data were analysed by one way ANOVA followed by Tukey's multiple comparision test.

Graph No. 10 Effect of Diosmin on Biogenic amines (Dopamine, 3,4- Dihydroxy Phenyl acetic acid, Norepinephrine and epinephrine) of Frontal cortex in Arsenic trioxide induced neurotoxicity in rats



N = 6; values were expressed as Mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 when compared to normal control; #p<0.05, #p<0.01, ###p<0.001 when compared to arsenic trioxide treatment group. Data were analysed by one way ANOVA followed by Tukey's multiple comparision test.

Graph No. 11 Effect of Diosmin on Biogenic amines (Serotonin and Homovanilic acid) of Frontal cortex in Arsenic trioxide induced neurotoxicity in rats



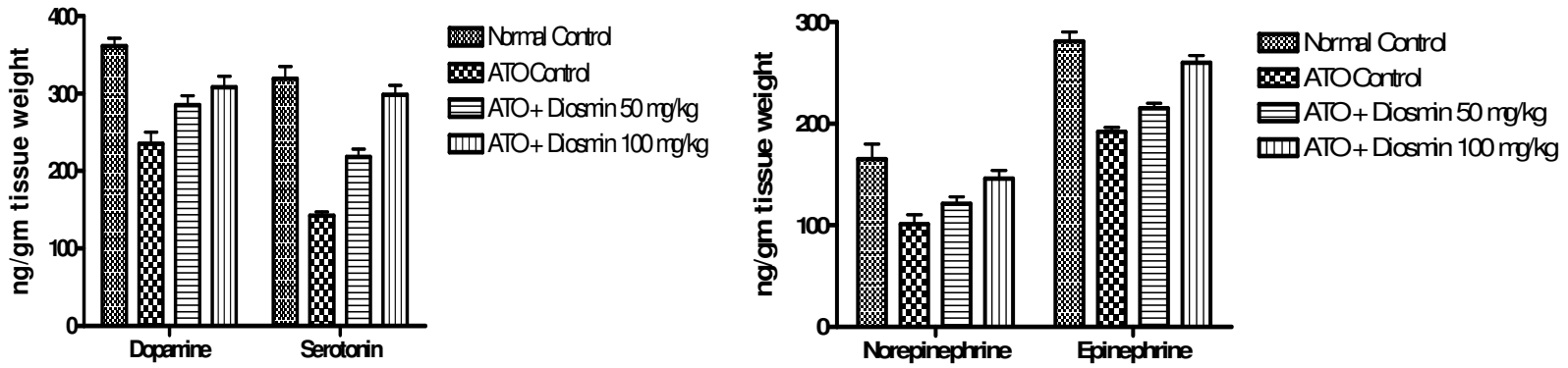
N = 6; values were expressed as Mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 when compared to normal control; #p<0.05, #p<0.01, ###p<0.001 when compared to arsenic trioxide treatment group. Data were analysed by one way ANOVA followed by Tukey's multiple comparision test.

Table No. 8 Effect of Diosmin on Biogenic amines of Hippocampus in Arsenic trioxide induced neurotoxicity in rats

S. No	Treatment	Biogenic amines (ng/gm tissue weight)					
		Dopamine	**Norepinephrine**	**Epinephrine**	**serotonin**	**3, 4 dihydroxy phenyl acetic acid**	**Homovanilic acid**
1.	**Normal control**	361 ± 10.02	165 ± 14.81	281 ± 9.36	319 ± 15.74	41.59 ± 7.14	37.47 ± 4.89
2.	**Arscenic trioxide (5mg/kg)**	235 ± 15.02***	101 ± 9.48***	192 ± 4.42***	142 ± 5.20***	25.42 ± 4.56***	19.42 ± 3.72***
3.	**Arscenic trioxide (5mg/kg) + Diosmin (50mg/kg)**	285 ± 12.14$^{\#\#}$	121 ± 7.01*$^{\#}$	215 ± 5.03$^{\#}$	218 ± 10.03$^{\#\#}$	29.52 ± 5.03$^{\#}$	24.91 ± 4.52$^{\#}$
4.	**Arscenic trioxide (5mg/kg) + Diosmin (100mg/kg)**	308 ± 14.12$^{\#\#\#}$	146 ± 8.23$^{\#\#}$	260 ± 7.16$^{\#\#\#}$	298 ± 12.42$^{\#\#\#}$	36.42 ± 4.49$^{\#\#}$	32.98 ± 3.44$^{\#\#\#}$

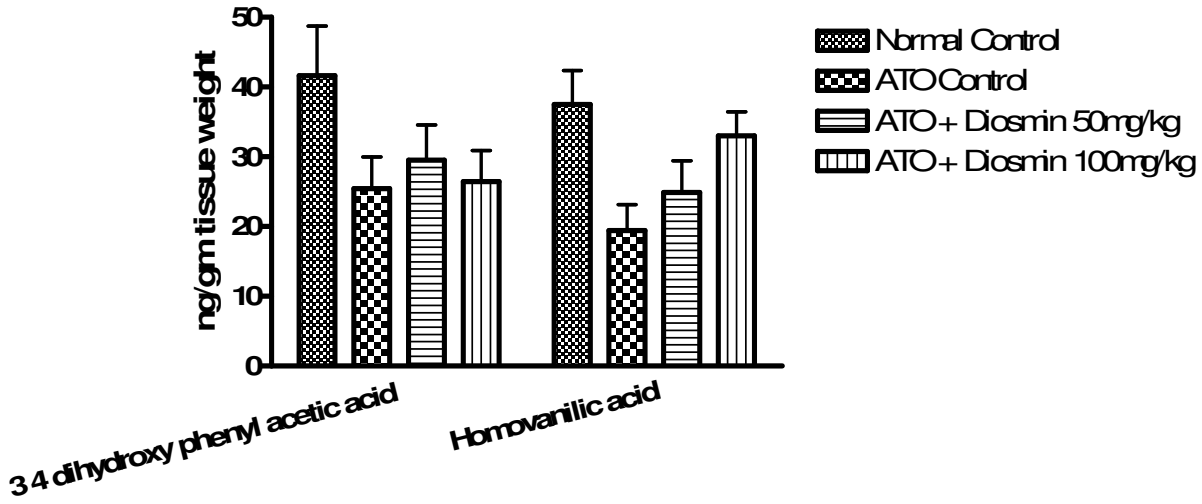
N = 6; values were expressed as Mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 when compared to normal control; #p<0.05, #p<0.01, ###p<0.001 when compared to arsenic trioxide treatment group. Data were analysed by one way ANOVA followed by Tukey's multiple comparision test.

Graph No. 12 Effect of Diosmin on Biogenic amines (Dopamine, Serotonin, Norepinephrine, Epinephrine) of Hippocampus in Arsenic trioxide induced neurotoxicity in rats



N = 6; values were expressed as Mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 when compared to normal control; #p<0.05, #p<0.01, ###p<0.001 when compared to arsenic trioxide treatment group. Data were analysed by one way ANOVA followed by Tukey's multiple comparision test.

Graph No. 13 Effect of Diosmin on Biogenic amines (3,4- Dihydroxy Phenyl acetic acid and Homovanilic acid) of Hippocampus in Arsenic trioxide induced neurotoxicity in rats



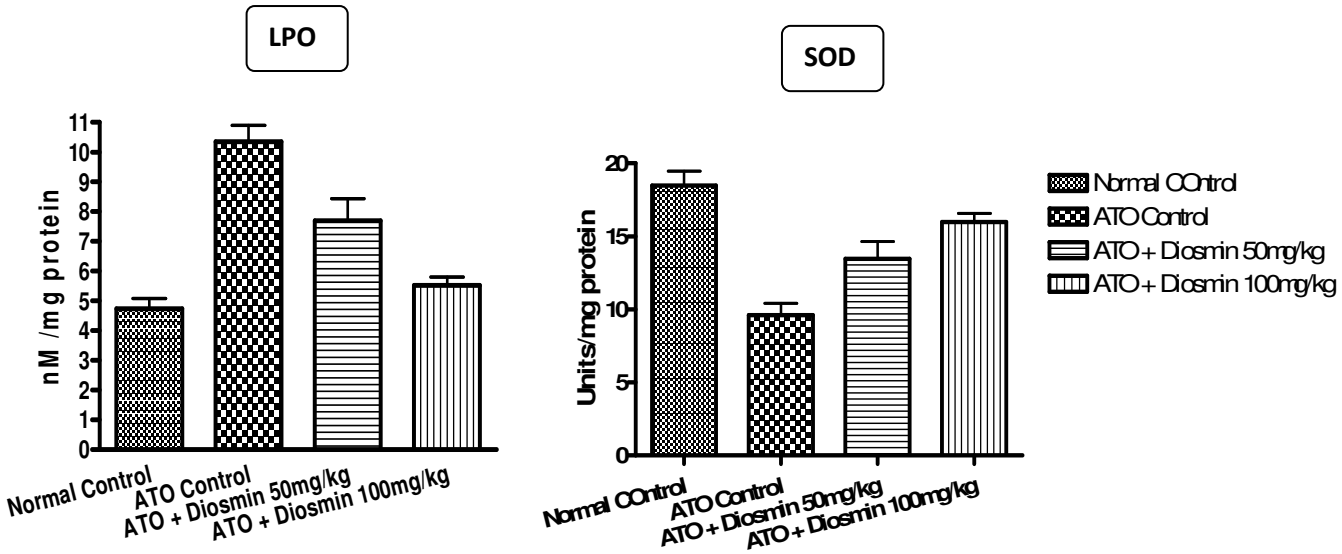
N = 6; values were expressed as Mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 when compared to normal control; #p<0.05, #p<0.01, ###p<0.001 when compared to arsenic trioxide treatment group. Data were analysed by one way ANOVA followed by Tukey's multiple comparision test.

Table No. 9 Effect of Diosmin on Brain tissue Antioxidants in Arsenic trioxide induced neurotoxicity in rats

S.No	Treatment	LPO (nM /mg protein)	SOD (Units/mg protein)	GSH (µg of GSH/mg protein)	CAT (µ moles of H_2O_2 consumed/min/mg)
1	**Normal control**	4.73 ± 0.34	18.47 ± 1.00	142.5 ± 3.22	1.49 ± 0.04
2	**Arscenic trioxide (5mg/kg)**	10.34 ± 0.55$^{###}$	9.60 ± 0.82$^{###}$	71.83 ± 4.67$^{###}$	0.53 ± 0.02$^{###}$
3	**Arscenic trioxide (5mg/kg) + Diosmin (50mg/kg)**	7.69 ± 0.74**	13.46 ± 1.18*	110.2 ± 10.80**	0.80 ± 0.01**
4	**Arscenic trioxide (5mg/kg) + Diosmin (100mg/kg)**	5.52 ± 0.28***	15.97 ± 0.61***	131.30 ± 3.16***	1.09 ± 0.06***

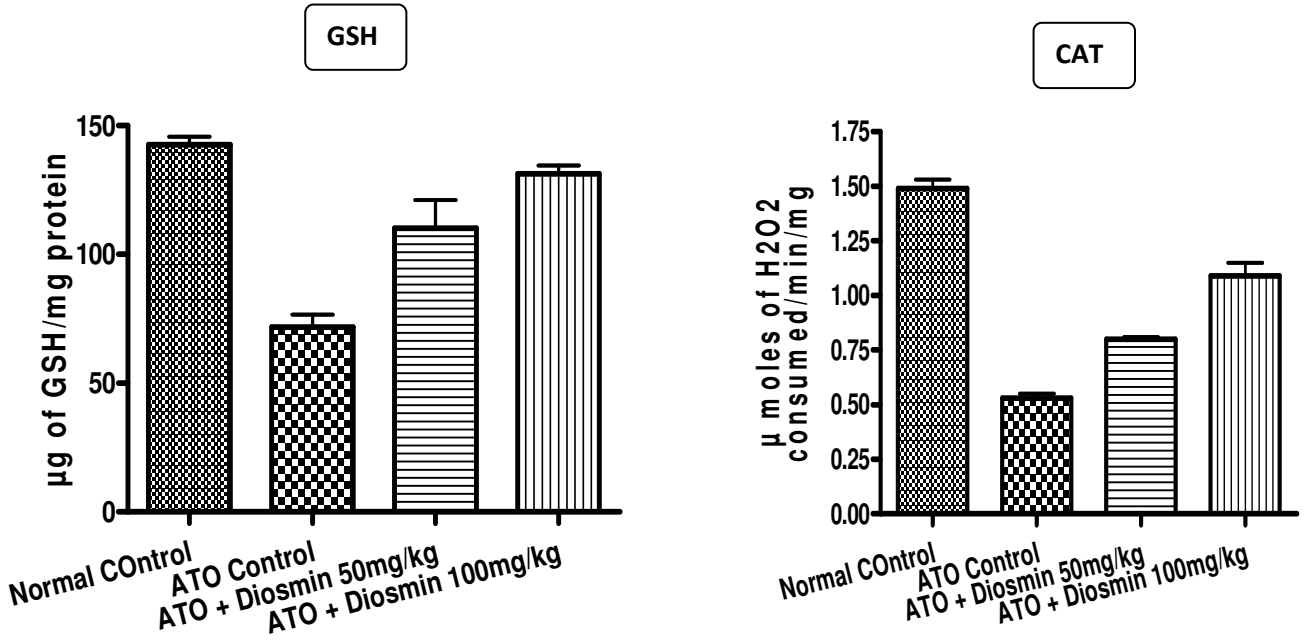
N = 6; values were expressed as Mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 when compared to normal control; #p<0.05, #p<0.01, ###p<0.001 when compared to arsenic trioxide treatment group. Data were analysed by one way ANOVA followed by Tukey's multiple comparision test.

Graph No. 14 Effect of Diosmin on Brain tissue Antioxidants in Arsenic trioxide induced neurotoxicity in rats



N = 6; values were expressed as Mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 when compared to normal control; #p<0.05, #p<0.01, ###p<0.001 when compared to arsenic trioxide treatment group. Data were analysed by one way ANOVA followed by Tukey's multiple comparision test.

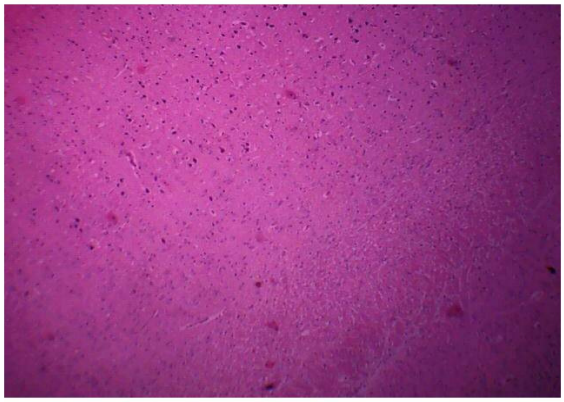
Graph No. 15 Effect of Diosmin on Brain tissue Antioxidants in Arsenic trioxide induced neurotoxicity in rats



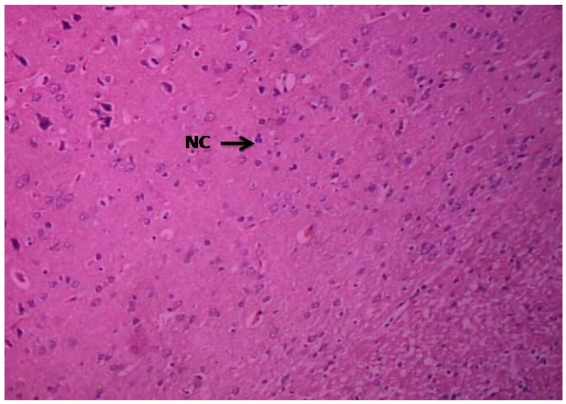
N = 6; values were expressed as Mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 when compared to normal control; #p<0.05, #p<0.01, ###p<0.001 when compared to arsenic trioxide treatment group. Data were analysed by one way ANOVA followed by Tukey's multiple comparision test.

7. HISTOPATHOLOGY

Photo micrograph of Brain showing normal cytoarchitecture of normal control animals:

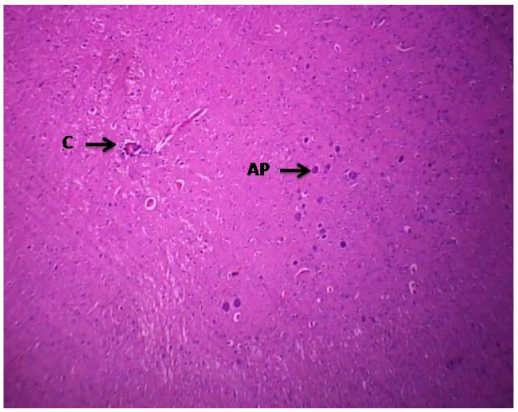


Normal Brain 4X

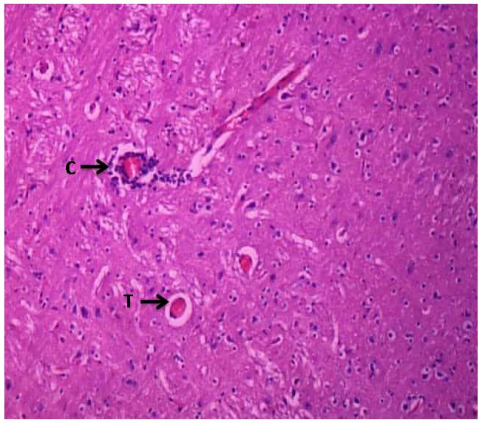


Normal Brain 10X

Photo micrograph of Brain showing degenerated neural cells of ATO (5 mg/kg) control animals:

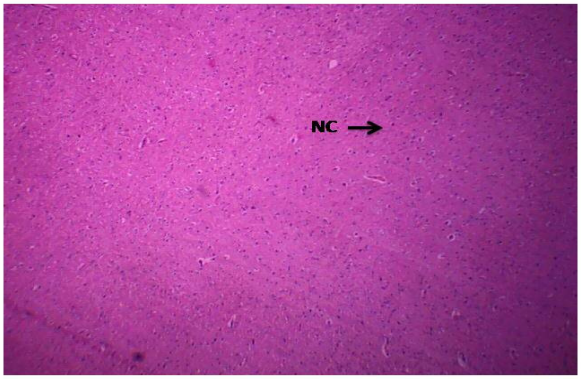


Control Brain 4X

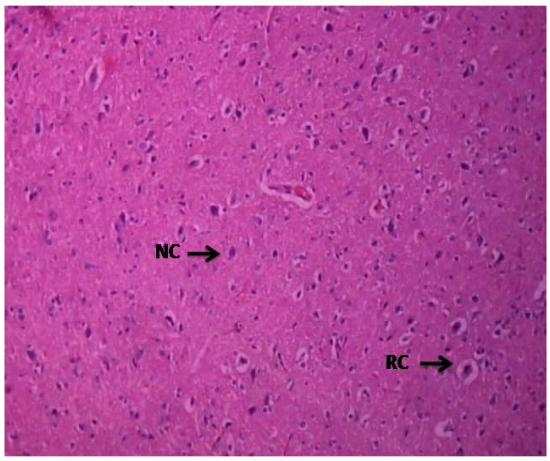


Control Brain 10X

Photo micrograph of Brain showing normal cytoarchitecture and recovering cells of animals treated with ATO (5 mg/kg) + Diosmin (50 mg/kg)

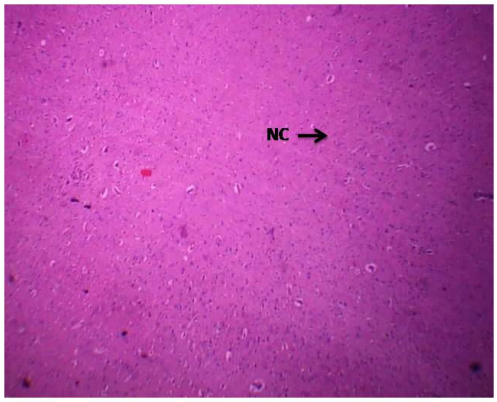


Test-1 Brain 4X

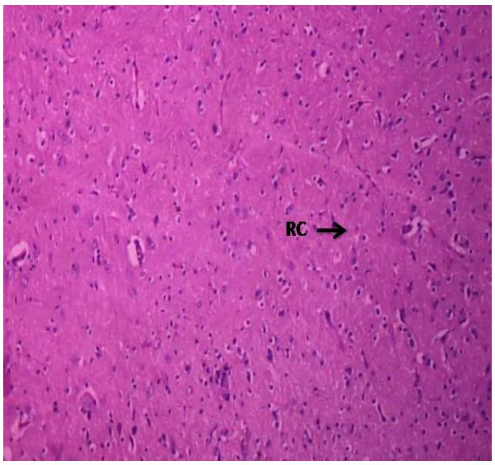


Test-1 Brain 10X

Photo micrograph of Brain showing normal cytoarchitecture of animals treated with ATO (5 mg/kg) + Diosmin (100 mg/kg)



Test-2 Brain 4X



Test-2 Brain 10X

8. DISCUSSION

Neuroprotective effect of Diosmin was evaluated using Arsenic trioxide as inducing agent in wistar rats. Statistical analysis of change in body weights, weights of brain, serum biochemical parameters, biogenic amines, serum and brain AchE levels and brain tissue antioxidant profile evaluation was done using instant prism graph pad (Ver 4.0).

Results revealed that treatment with ATO showed a significant decrease in plasma total protein (TP) and albumin (A). On the other hand, treatment with ATO caused a significant increase in the concentrations of plasma glucose, urea, creatinine and bilirubin as compared to control. The decline in plasma total protein due to treatment with ATO was mainly due to the decrease in albumin fraction.[132, 114, 115]

The inhibitory effect of ATO on protein profile showed that arsenic inhibited the ability of glucocorticoid and its receptor to turn on genes normally. The glucocorticoid hormonal system plays an important role in protein metabolism. The reduction in plasma protein in animals exposed to environmental pollutants could be attributed to changes in protein and free amino acid metabolism and their synthesis in the liver.

In addition, the protein depression in the blood was also reported to be mainly due to excessive loss through nephrosis. Also, may be due to reduced protein synthesis or increased proteolytic activity or degradation. The observed decrease in plasma proteins could be also attributed in part to the damaging effect of ATO on liver cells.

The increased glucose production and decreased glucose utilization would lead to hyperglycemia. Oxidative stress (OS) has been suggested as a major pathogenic link to both insulin resistance and beta cell dysfunction. [114, 115]

Oxidative stress causes structural damages to the pancreatic islets with the formation of amyloid proteins, which not only prevents the release of insulin into the circulation, but also destroys the insulin-secreting beta cells insidiously after prolonged exposure to arsenic.

The elevation in plasma urea level in ATO treated rats is considered as a significant marker of renal dysfunction. The blood urea becomes raised when the kidney tubules are prevented from removing the urea and other waste products from the blood. Elevated blood urea

is correlated with an increased protein catabolism in mammalian body or from more efficient conversion of ammonia to urea as a result of increased synthesis of enzyme involved in urea production.

Also, the high levels of blood urea results from either increased breakdown of tissue, dietary or impaired excretion. Moreover, the increase in urea concentrations in plasma of animals treated with ATO may be due to its effect on liver function, as urea is the end-product of protein catabolism and this is confirmed by the decrease in plasma.

The present study indicated that treatment with ATO caused significant increase in plasma creatinine and urea. Low clearance values for creatinine and urea indicate diminished ability of the kidneys to filter these waste products from the blood and excrete them in the urine.

Thomas et al. found that the induction rate in serum bilirubin was associated with free radical production. The lipophilic reactive oxygen species act directly on bilirubin, leading to its oxidation to biliverdin. Radicals derived directly from arsenic or during its metabolism, and they play an important role in the nephrotoxicity by increasing lipid peroxidation.[132]

The increase in plasma bilirubin (hyper-bilirubenimia) may be resulted from decreased liver uptake, conjugation or increased bilirubin production from hemolysis. Also, the elevation in plasma bilirubin concentration could be due to the onset of periportal necrosis. [114, 115, 133]

The present data indicated that plasma TL, cholesterol, TG and LDL-c were significantly increased by ATO treatment, while HDL levels were decreased.

The present results showed that treatment with Diosmin alone caused a significant decrease in glucose, urea, creatinine, TL, cholesterol, TG and LDL-c, but did not cause changes in TP, A, G and bilirubin. Diosmin treatment significantly attenuated the ATO mediated increase in plasma glucose, urea, and creatinine, and decrease in total protein and albumin.

This effect may be related to the antioxidant properties of Diosmin, since it has been found that ROS may be involved in the impairment of glomerular filtration rate.

The decrease in glucose level of rats treated with Diosmin reported that daily intake reduce the blood sugar level.

Acetyl cholinesterase (AChE) is one of many important enzymes needed for the proper functioning of the nervous systems of humans, other vertebrates and insects.[133]

The data obtained from this study clearly show that arsenic trioxide significantly decreased the activity of serum acetyl cholinesterase. The results agree with previous studies that demonstrated a decreased activity of acetyl cholinesterase in neuroblastoma cells of mice, in rat whole brain and in two models of fish.

The inhibition of acetyl cholinesterase by arsenic trioxide is a puzzling phenomenon because AChE does not contain the structural features usually associated with the inhibition of enzymes by arsenic.

Trivalent arsenic compounds are potent inhibitors of a number of enzymes but the mechanism of this inhibition is the reaction of the arsenical with free sulfhydryl groups, notably those of reduced lipoic acid, to form cyclic thio-arsenite diesters. The enzyme has been found to contain cysteine only in the form of disulfide bridges and not as free thiol.

Biogenic amines are important regulators of various physiological and pharmacological functions in the biological system. Fluctuations in the levels of biogenic amines may affect the signaling process and behavioral functions. Alterations in the levels of brain catecholamines, 5-HT and their metabolites have been observed in chemical-induced neurodegeneration and also in neurodegenerative diseases.[140]

Although a number of studies have been carried out to investigate the role of biogenic amines including DA, NE, EPN and 5-HT and their metabolites in arsenic-induced neurotoxicity, consistent changes have not been observed.

In our study upon given with ATO showed a significant reduction in the biogenic amines (dopamine, NE, Epinephrine, 5 HT, Homovanilic acid and 3, 4 – dihydroxy phenyl acetic acid) indicating the reduced neuronal function of different regions of brain (Corpus striatum, frontal cortex, hippocampus). Treatment with Diosmin in rats revealed a successful recovery in the production of biogenic amines replacing the active neuronal functions.

In the present study, various antioxidant parameters were assessed in the brain at the end of the study.

The thiobarbituric acid reacting substance assay is used as an indicator of lipid peroxidation and levels of free radicals. The assay is based on the reactions of thiobarbituric acid with malondialdehyde produced during lipid peroxidation.

The results showed that the effect of ATO was significant in increasing in malondialdehyde when compared with the normal group. Treatment with Diosmin showed significant decrease in malondialdehyde when compared to ATO treated group.

Superoxide dismutase is an important endogenous antioxidant and prevents production of free radicals. The decrease in superoxide dismutase is observed in ATO treated group compared normal group. The results from treatment with Diosmin showed that a significant rise in SOD levels revealed the protective effect of the Diosmin.

Reduced Glutathione (GSH) is one of the primary endogeneous antioxidant defense systems in brain, which removes hydrogen peroxides and lipid peroxidase. Decline in GSH levels could either increase or reflect oxidative state.

The decrease in GSH is observed in ATO treated group compared to normal group. Treatment with Diosmin brought the GSH to the normal levels when compared to ATO treated animals.

An increase in production of catalase leads to the accumulation of hydrogen peroxidase, which is converted to hydroxyl radicals that produced deleterious effect on brain. Catalase decomposes hydrogen peroxidase and converts it to water and diatomic oxygen, whereas superoxide dismutase generates H_2O_2 from free radicals.

The decrease in H_2O_2 consumed is observed in ATO treated group compared to normal group. The results from the Diosmin treatment showed that catalase levels were significantly recovered when compared to ATO treated group, and no significant difference was observed when compared to normal control.

Histopathological studies of the normal control brain showed normal cytoarchitecture in brain. In arsenic trioxide treated group of animals showed the congestion of degenerative changes due to decrease in the number of neural cells in brain.

Animals treated with Diosmin at a dose of 50 mg/kg showed regenerative changes in brain. Interestingly high dose of Diosmin 100 mg/kg treated rats showed almost normal cytoarchitecture of brain.

Despite its protective actions on the central nervous system, there have been very limited studies of the effects of Diosmin on the brain and behaviour. Therefore, the present study provided new insights on the neurotoxicity of arsenic.

9. CONCLUSION

In summary, the results of the present study suggest that arsenic neurotoxicity in rats initiated peroxidative reactions in membrane lipids of the brain.

The present study demonstrates the inhibitory effect of Diosmin pretreatment on the brain oxidative stress in an *in vivo* model. Histopathological evaluation revealed the neuroprotective effect of Diosmin mainly at a dose of Diosmin 100 mg/kg.

Treatment with Diosmin revealed a significant amelioration in arsenic induced neurotoxicity showing its protective effect by improving the Biogenic amines in various regions of brain and the AchE levels in serum and brain.

These findings derive the significance of Diosmin in ameliorating the oxidative stress and neuroinflammation which are important contributors in the pathogenesis of several neurodegenerative disorders.

Furthermore, it is implicated that arsenic is a de-myelinating agent. And may alter neuronal functions followed by CNS dysfunctions.

Much remains to be learned about this ancient neurotoxicant. It is planned in future to study the effects of arsenic in brain with respect to myelin structure and functions, DNA and RNA levels and to seek a correlation with oxidant stress, and to estimate the levels of antioxidant defence system enzymes.

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